

Endocrinology of photoperiodic diapause  
induction in two species of Diptera

by

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God in his wisdom

created the fly

and then forgot

.

to tell us why

Ogden Nash



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## Abstract

No difference was noted in the timing of ovarian, embryonic and feeding larval development in the flesh-fly *Sarcophaga argyrostoma* (Robineau Desvoidy) in diapause and non-diapause inducing photoperiods. Prior to the onset of the larval post-feeding stage, no differences were noted between three selected strains of *Sarcophaga*. After wandering however, the SLOW strain wanders for a longer period, and the FAST strain, a shorter period, than the STOCK strain from which they were derived. In all cultures, those in diapause inducing short-day photoperiods delay pupariation for longer than those in non-diapause inducing photoperiods. The effects of the length of the wandering period and of crowding on subsequent diapause incidence are examined. The development of an artificial uterus in which embryos isolated from the adults could be subjected to different light/dark regimes suggests that maximum photoperiodic sensitivity occurs during embryonic and larval development. No maternal influence on diapause induction was observed in this species of *Sarcophaga*.

In mixed age cultures, the initiation of larval wandering occurs as a gated circadian rhythm. In light/dark (LD) cycles, most of this activity occurs in the dark, except in very short nights, or in certain phase relationships between the rhythm and light cycle. When transferred from series of LD cycles into continuous darkness (DD), cultures show a weakly persistent free-running rhythm with a period of about 21h. However, after transfer of first instar larvae from continuous light (LL) to DD, no such rhythm is observed. In contrast to larval exodus, formation of puparia and larviposition can occur at any stage of the LD cycle. The physiological mechanisms underlying this gated exodus behaviour, and its possible selective advantages are discussed.

No differences were observed between the rates of development of larvae and pupae from diapause and non-diapause destined lines of *Sarcophaga* except that those destined for diapause have a longer post-feeding, wandering, larval phase associated with a lower haemolymph ecdysteroid titre, as measured by radioimmunoassay. Following pupariation, both cultures show a high haemolymph titre associated with larval/pupal apolysis. The developing culture displays an ecdysteroid peak at 72h post-pupariation which may be involved with pupal/adult apolysis and the initiation of pharate adult development. This peak is reduced in the diapause destined culture. Following the initiation of pharate adult development, there is a very large peak at 85-90h. Those pupae entering diapause display very low titres presumably as a result of the failure of the brain/prothoracic gland axis to release ecdysone. There are no quantitative or qualitative differences between the titres of specific ecdysteroids in the prepupae of the two lines as determined by reverse phase high performance liquid chromatography. A preliminary examination of the levels of free and conjugated ecdysteroids has provided the basis for proposing a mechanism of ecdysone metabolism in this insect.

The *in vitro* activation of isolated prothoracic glands from *Sarcophaga* was accomplished with extracts of prepupal brains. The action of putative prothoracicotrophic hormone (PTTH) could be mimicked by the use of cAMP analogues and a phosphodiesterase inhibitor, suggesting the involvement of a cyclic nucleotide mediated secondary messenger system. This was further supported by the removal of calcium ions thereby lowering the effect of the PTTH extracts *in vitro*. Cyclic GMP and its derivatives had no effect on ecdysone synthesis. The PTTH extract from *Sarcophaga* was found to be equally effective at stimulating the production of ecdysone by isolated *Calliphora vicina* (Meigen) ring glands.

The nature and time course of ring gland competency in diapause in both *Sarcophaga* and *Calliphora* was investigated. As the *Sarcophaga* population entered pupal diapause at 96h post-pupariation at 18°C, the prothoracic glands became refractory to PTTH, taking 24h to lose their competency to respond to this hormone. The levels of ecdysone produced were below the limits of detection of the radioimmunoassay used. The glands could not be stimulated by 10mM cyclic nucleotides indicating that the block to PTTH action occurred beyond the proposed stage of cyclic nucleotide mediation. The *Calliphora* population, which has a larval diapause, took six days to reach this refractory state. Their prothoracic glands continued to produce ecdysone at the basal rate throughout diapause and this basal rate could not be altered by the application of PTTH or cyclic nucleotides. Following a temperature increase from 11°C to 25°C, all diapausing *Calliphora* had regained prothoracic gland competency within 24h and had pupariated within 36h. When isolated brain/ring gland complexes from diapausing *Calliphora* were incubated at 25°C, no recovery of ring gland competency was noted, indicating the requirement for *in vivo* reactivation after diapause. The levels of PTTH in pre-diapause and day 35 post-pupariation diapause brains of *Sarcophaga* were shown to be similar to those of non-diapause destined pre-pupal brains. A discussion of the involvement of neuropeptides and other neurotransmitters in the regulation of PTTH release is presented.

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## GENERAL INTRODUCTION

Large seasonal variations in temperature, humidity and food availability occur in many regions of the world, particularly at higher, temperate latitudes. To cope with periods when conditions are unsuitable for continuous growth and development, many insects enter a diapause state in which growth is arrested and metabolism reduced. The term diapause, first used by Wheeler (1869) to describe a specific stage in the embryogenesis of a grasshopper, was redefined as a condition of arrested growth in any developmental stage of an organism by Henneguy (1904). This has since been divided into quiescence, when growth is interrupted in direct response to unfavourable environmental conditions and resumed as soon as these conditions are removed, and true diapause which results from factors which are not themselves adverse but signal the impending onset of unfavourable conditions. True diapause is often terminated long after the disappearance of the adverse conditions. This implies a degree of endogenous control rather than the exogenous control exerted in quiescence.

Muller (1970) reclassified diapause into three categories: parapause, eudiapause and oligopause. Parapause is equivalent to obligatory diapause, in which induction is completely independent of environmental conditions. An example is the spruce budworm *Choristoneura fumiferana* (Harvey, 1957), in which every individual in every generation undergoes diapause as part of its life cycle. The terms eudiapause and oligopause divide facultative diapause which may or may not occur in a particular generation into two classes depending upon the environmental conditions that the population is exposed



to. In eudiapausing species, diapause can be induced by one factor (for example, photoperiod) but can be terminated by changes in the level of another (for example, temperature). This type of diapause is found in the flesh-fly *Sarcophaga argyrostoma* (Robineau-Desvoidy) and in the blowfly *Calliphora vicina* (Meigen), the two species on which this thesis is based. In oligopause, both induction and termination are under the control of the same factor (for example photoperiod). This division of diapause may however not be entirely valid since on further investigation, temperature has been shown to influence diapause in species previously thought to possess a photoperiodically induced oligopause, for example, the giant silkworm *Antheraea pernyi* (Mansingh and Smallman, 1971). Tanaka (1944) had shown that temperature had no effect on the termination of photoperiodically induced diapause in this species. Mansingh and Smallman (1971) however, showed that termination was indeed effected in unchilled pupae at 32°C even in short-day photoperiods. Similar doubt was cast on the concept of oligopause by Hodek (1968) working on the Linden bug *Pyrrhocoris apterus* in which once again, diapause could be broken by high temperatures even in short-day photoperiods. The work presented in this thesis will concentrate on the eudiapause of *Sarcophaga* and *Calliphora* which henceforth will simply be termed diapause.

Diapause in *Sarcophaga argyrostoma* as in many other insect species is induced by shortening photoperiod in conjunction with lower temperatures. The use of photoperiod as an inducing agent has presumably evolved because daylength is a very precise and reliable indicator of the seasons and therefore of probable environmental conditions. Other potential criteria may include temperature, humidity, and food availability. These however, can be subject to large local variations thereby reducing their effectiveness as predicting agents.

Extreme seasonal fluctuations in photoperiod are found at high latitudes whilst on, or close to the equator, photoperiod remains close to a 12h light: 12h dark (LD12:12) regime at all times of the year. With increasing latitude, summer daylength increases whilst mean summer temperatures decrease. Bradshaw (1976) found that the length of the growing season, defined as the mean number of frost-free days, also decreases with increasing latitude. In temperate regions therefore, the summer provides less time for growth and development thereby restricting the number of possible generations per year. Northerly populations require an earlier onset of diapause to avoid unfavourable conditions than do more southerly populations. Some equatorial regions however may be subject to summer droughts. A species with a wide latitude range may therefore possess both a summer and a winter diapause. Masaki (1980) demonstrated that the cabbage moth *Mamestra brassicae* has a winter diapause in its northerly populations and a summer diapause further south in the sub-tropics.

For diapause to be induced by photoperiod, certain 'decisions' regarding the day length must be made. The daylength which delimits those individuals which enter diapause and those which undergo continuous development is known as the critical photoperiod. If groups of insects from one population are experimentally exposed to a range of photoperiods under controlled conditions of temperature *etc.*, the critical photoperiod is the point at which 50% enter diapause. The shape of the resulting curve, known as a photoperiodic response curve (PPRC), depends upon whether the species has a summer or winter diapause and upon the temperature at which the cultures were maintained. A summer active species with a winter diapause will possess a long-day response. Short-day species are active during the shorter days of winter. *Mamestra* has a PPRC with two critical photoperiods and will only undergo

continuous development in photoperiods between these points (Masaki,1980).

As might be expected, the critical photoperiod can vary within a species depending upon latitude. Danilevskii *et al.* (1970) proposed the empirical rule that the critical photoperiod in genetic sub-populations lengthens by 1 to 1.5h per 5° rise of latitude. Bradshaw (1976) further noted that with the mosquito *Wyeomyia smithii*, which displays a facultative winter larval diapause, a change in 1° in latitude was equivalent to a 142m rise in altitude in its effect on the critical photoperiod. Further south and within the tropics, daylength changes become smaller and the role of photoperiod on the seasonal patterns become less clear. The locust *Nomadacris septemfasciata* is able to sense changes even within 10° of the equator (Norris,1959,1962). Some species rely on environmental cues other than photoperiod. Low temperature was able to induce diapause in several flesh-fly species living close to the equator in East Africa (Denlinger,1974,1978) where daylength changes by as little as 7 minutes throughout the year. Photoperiod not surprisingly had no effect on diapause induction in these examples. The effects of geographical variations on diapause induction and other photoperiodic responses have been reviewed by Saunders (1982).

The role of temperature on diapause induction is highly complex and variable, depending upon whether a thermoperiod (temperature cycle) or constant temperature is experienced. Saunders (1973) noted that short-day thermoperiods in constant darkness (DD) could induce diapause in the parasitic wasp *Nasonia vitripennis*, and that thermoperiods could enhance the effects of coincidental short-day photoperiods. Similar responses were noted in the southwestern cornborer *Diatraea grandiosella* (Chippendale and Reddy,1973) in which maximum diapause incidence occurred in response to the coincidence of

a long night with the cool portion of a thermoperiod. In the Colorado beetle *Leptinotarsa decemlineata*, the critical photoperiod increased by 2h for each 5°C fall in aperiodic temperature (Danilevskii,1971). In contrast, the critical photoperiod in the tobacco hornworm *Manduca sexta* only increased by 15 minutes in response to a temperature decrease from 30°C to 26°C (Bell *et al.*,1975). This and other similar examples indicate a degree of temperature compensation of the critical photoperiod in some species. Danilevskii (1965) established a wide range of temperature compensation in the cabbage white butterfly *Pieris brassicae*. Similar compensation was noted by Saunders (1971) up to 18°C in *Sarcophaga argyrostoma* maintained in constant light as embryos, and thereafter in short-days. Above this temperature, diapause incidence was found to be low even in diapause inducing conditions. If embryos are reared in short-day photoperiods, diapause is possible up to higher temperatures, as will be seen later.

This thesis will examine various aspects of diapause in the flesh-fly *Sarcophaga argyrostoma* and attempt to discuss these in the context of the existing literature. Chapter 1 concentrates on the ovarian, embryonic and larval development under diapause and non-diapause inducing conditions. The growth rates of diapause and non-diapause induced lines of three selected strains, which vary only in the length of the post-feeding wandering stage, will be investigated with particular reference to the effects of crowding on the timing of puparium formation and subsequent diapause incidence. In conjunction with these investigations, a study of the timing of the photoperiodically sensitive stage of this species and the existence or otherwise of a maternal influence will be presented. These aspects will be presented and discussed in the light of a literature review covering Dipteran embryogenesis, autogeny, the photoperiodically sensitive stages of insect life

cycles, the diapausing stage, maternal influences on diapause induction and the photoperiodic counter theory which attempts to account for the need for several inductive cycles before diapause is induced.

Following on from the work on the post-feeding stage of larval development, the role of the circadian clock system in the timing of several developmental events will be examined in chapter 2. The length of the post-feeding stage will be discussed in the context of the timing of larval wandering from the food and puparium formation. One of the functions of the work presented in these first two chapters is to provide a series of frameworks within which the major portions of the thesis, chapters 3 and 4, can be designed and discussed.

Chapter 3 begins the examination of the endocrinology of diapause induction in *Sarcophaga*. Data will be presented with a review of the known endocrinology of normal and diapause development in insects in general and *Sarcophaga* in particular. Using a strain of *Sarcophaga* selected on the basis of work in chapter 1, development in both long and short-day photoperiods will be examined. Differences in haemolymph ecdysteroid titres between diapause and non-diapause destined cultures in larval and pupal development will be examined as a means of determining potential mechanisms of diapause induction. Data on specific ecdysteroid titres will be presented to examine if differences here could affect or be a result of induction. The role of prothoracic glands (which are the primary source of ecdysone) and brains of *Sarcophaga* in both diapause and non-diapause development will be examined *in vitro* in chapter 4. These studies will be presented with a review of prothoracic gland physiology in both normal and diapause development. In addition to *Sarcophaga* which has a pupal diapause stage, similar

investigations into prothoracic gland function in *Calliphora vicina* which has a larval diapause will be presented. Differences between the responses of diapause and non-diapause induced prothoracic glands in both species will be examined. Changes in these functions will be studied in both species as they enter and recover from diapause.

Each chapter will comprise a general introduction in which the relevant literature to the objectives of the chapter will be presented. The data will be discussed in the context of this literature with particular reference to diapause induction and termination in the species under examination.

## General Methods

The strain of the flesh fly *Sarcophaga argyrostoma* (Robineau-Desvoidy) used in the course of the investigations described in this thesis has been maintained in the Zoology Department, Edinburgh University since 1961. Prior to this date, its history is unknown. The strain of the blow fly *Calliphora vicina* (Meigen) used in chapter 4 was collected from the Edinburgh area in 1984.

### Light and temperature conditions

#### Stock cultures

Stock cultures were kept in thermostatically controlled constant temperature rooms at  $25 \pm 1^\circ\text{C}$ . Illumination was by overhead fluorescent striplights controlled where applicable by Venner time switches. Most of the stock cultures were maintained under constant light (LL), except when a diapausing culture was required when they were kept under LD 12:12.

#### Experimental cultures

Most experiments were conducted in walk-in constant temperature cabinets maintained at either  $25 \pm 0.5^\circ\text{C}$  or  $20 \pm 0.5^\circ\text{C}$ . Cultures of larvae or adults were placed in light-proof wooden boxes within the constant temperature cabinet, each of which was fitted with a water jacketted 4W fluorescent light source (Philips) controlled by individual Venner time switches. Temperatures in the boxes and within the cultures were measured by a Rustrak temperature probe. The lights-on portion of the LD cycle did not cause a significant temperature rise, but temperatures within the cultures rose  $2\text{--}3^\circ\text{C}$  above

ambient towards the end of the larval feeding phase.

### Stock cultures

Adult flies were maintained in breeding cages (60x50x45cm) constructed from a metal frame on a solid metal base. Each cage was covered with a tube of fine white gauze knotted at each end and the base was lined with white paper. The flies were provided with sugar and water *ad libitum*. Fresh meat (beef muscle) was supplied daily to provide both the protein source required for anautogenous ovarian growth (see chapter 1) and a site for larviposition.

Larvae from the stock cages were reared at 25°C. Cultures were established by transferring pieces of meat carrying newly deposited larvae to plastic dishes containing about 400ml of a dried milk-yeast-agar synthetic medium (described below). The cultures contained between 400–500 larvae deposited by a number of females from within the stock population. These plastic dishes were placed within plastic mouse trays containing fine sawdust to a depth of about 1cm, and covered with a fine gauze lid. After 5–6 days, the mature 3rd instar larvae left the food and wandered into the sawdust to form puparia (Saunders, 1971). The time taken to form the puparia was different for the three strains of *Sarcophaga* used in this investigation (see chapter 1). Newly formed puparia were then sieved from the sawdust and allowed to dry for three days at room temperature. The cultures were covered with a cloth to protect them from the parasitic wasp *Nasonia vitripennis* (Hymenoptera; Pteromalidae) which parasitises the intrapuparial stages of cyclorrhaphous Diptera and is endemic in the Edinburgh laboratory. After drying, the pupae were stored in glass jars covered with paper towel lids in constant darkness at 22°C until the first adults emerged. The jars were then transferred to the stock cages and the newly emerged flies allowed to intermix with the existing stock.



**Experimental cultures**

These were established by placing the required number of larvae on pieces of meat on the artificial medium and rearing them under the conditions described in the various protocols of these investigations.

### Recipe for the synthetic medium

This medium was apparently devised by Peterson (1953), as cited by Haupt and Busvine (1968). In the present investigations, it was made up in 4l quantities.

### Ingredients

250g----dried milk powder (Millac, instant spray dried separated  
milk with added vegetable fat and vitamins A,C, and D)  
30g-----dried yeast (Distillers Co.) Autolysed yeast granules  
20g-----Davis Agar (Mackay and Lynn Ltd)  
4l-----water

### Method

The dried milk and the yeast were mixed in a plastic basin with 1.5l warm water to a smooth paste. The remaining water was heated to boiling and the agar added and dissolved. The heat was removed and the milk-yeast mixture added and stirred in before pouring the mixture into plastic dishes to set. The medium, once cool was stored at 4°C.

### Criteria for the determination of diapause and non-diapause development

*Sarcophaga argyrostoma* Fraenkel and Hsiao (1968a) demonstrated that pupae of *Sarcophaga argyrostoma* enter diapause after head evagination, before pupal-adult apolysis and the appearance of the developing antennae. All terminology used to describe the life cycle of *Sarcophaga* in this thesis is based on that recommended by Fraenkel and Bhaskaran (1973). Care has been taken to differentiate between the formation of the puparium (pupariation), which occurs when the larval cuticle hardens and tans at the end of the larval wandering period, and the actual larval/pupal apolysis (pupation), which occurs some 48h after pupariation at 25°C. The term 'moult' is used to include both apolysis (separation of the old cuticle from the underlying epidermis) and ecdysis (shedding of old cuticle). Thus ecdysis only occurs during the emergence of an adult fly from its puparium when both larval and pupal cuticles are shed simultaneously.

Newly formed pupae were given time to allow adult differentiation to take place in non-diapause destined individuals. When the first adults emerged from the puparia, about 12 days after pupariation, the cultures were placed at -10°C to kill the developers and the puparia opened to reveal the state of development inside. This was achieved by squeezing the puparium between finger and thumb to split the anterior cap of the puparium along the preformed eclosion lines level with the larval anterior spiracles. Although Fraenkel and Hsiao (1968a) state that the first sign of development (*i.e.* non-diapause) was the appearance of the white antennal discs beneath the transparent head cuticle, this was difficult to see. In this work, the appearance of eye and cuticle pigmentation and bristles on the head were the criteria used for non-diapause development. Pupae of the same age which showed

no sign of such pigmentation were assumed to be in diapause. Those insects which had died before development could be recognised by a discoloration or lack of any recognisable features within the puparium , as well as by their odour. Results are expressed as percentage diapause, *i.e.* the number of diapausing pupae divided by the total number of live pupae multiplied by 100.

*Calliphora vicina* Vinogradova and Zinojeva (1972) proposed that those larvae which had not formed puparia by 30 days after oviposition at 11°C could be assumed to be in diapause. This criterion is discussed in chapter 4 with reference to data presented there.

### **Stages of ovarian and embryonic development in *Sarcophaga argyrostoma***

The follicular stages of ovarian development have been based on Pappas and Fraenkel's (1977) modification of Harlow's (1956) scheme which was originally developed for the blowfly *Protophormia terrae-novae*, but is equally applicable to *Sarcophaga* spp., both having polytrophic ovarioles. These stages can be seen in figure 1 and are detailed below.

Stage 1: The primary follicle and germarium are indistinguishable, opaque and very small

Stage 2: The primary follicles are clearly distinguishable from the germarium. The follicle contains one egg cell and a number of nurse cells which are visible only under high magnification.

Stage 3: The primary follicles become enlarged and spherical with the nurse cells readily visible.

Stage 4 The egg cell gradually fills up with yolk at the start of vitellogenesis. As the follicle fills with yolk, the nurse cells gradually disappear. The follicle enlarges, partly due to developing polyploidy, and the secondary follicle becomes distinct from the germarium.

Stage 4 can be further subdivided according to Pappas and Fraenkel (1977) into 4a, 4b, and 4c. In summary,

4a, the follicle is one quarter full of yolk

4b, the follicle is half full of yolk

4c, the follicle is three quarters full of yolk

4c/5, the follicle is full of yolk, but unchorionated

5, the follicle is now chorionated ready for ovulation.

Following ovulation when the eggs are contained within the uterus, the development of trachea and spiracles can be followed and timed. The initiation of movement of the embryo within the chorion prior to larviposition can also be observed.

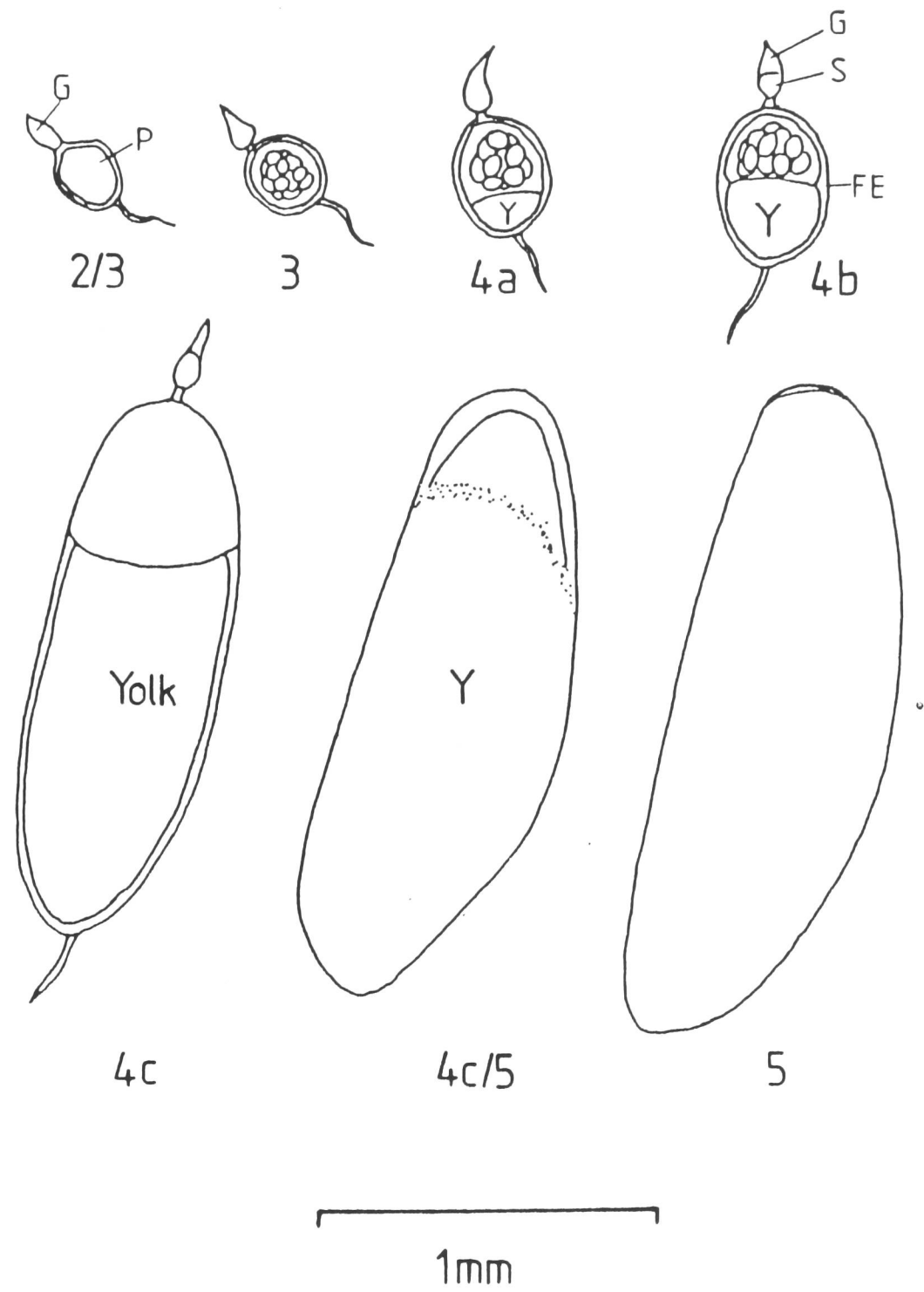


Figure 1: The development of ovarian follicles in the flesh fly *Sarcophaga argyrostoma*. P, primary follicle, G, germarium, FE, Follicular endothelium, S, secondary follicle. See text for details of developmental stages 2 to 5. After Kenny (1985).

## CHAPTER 1

Ovarian, embryonic and larval development in flesh flies  
under diapause and non-diapause inducing photoperiods



## OVARIAN, EMBRYONIC AND LARVAL DEVELOPMENT IN *SARCOPHAGA*

### UNDER DIAPAUSE AND NON-DIAPAUSE INDUCING PHOTOPERIODS

#### Introduction

This chapter will concentrate on the ovarian, embryonic and larval development of *Sarcophaga argyrostoma* under diapause and non-diapause inducing photoperiods. It will focus on any differences in growth rates between diapause and non-diapause destined lines, on the existence or otherwise of a maternal influence in diapause induction and on the development of an artificial uterus for the study of post-ovulation eggs and embryos *in vitro*. To provide a framework for later studies into diapause development in this thesis, the effects of photoperiod and overcrowding on the larval wandering period, puparium formation and subsequent diapause incidence will be examined.

*Sarcophaga*, in common with all Diptera, is characterised by a polytrophic ovariole in which a number of nurse cells (normally 15 in higher flies) together with the oocyte and a surrounding envelope of mesodermal cells make up a follicle destined to produce an individual egg (Engelmann, 1970). In *Drosophila*, oocyte differentiation takes place in the apical region of the germarium (see general methods section) which contains the stemline oogonia. Each of these gives rise to a cystoblast and another stemline oogonium during pupal and early adult life (King *et al.*, 1968). As the cystoblast passes down the

germarium, it divides four times thereby giving rise to the oocyte and the fifteen nurse cells. Mesodermal cells grow in and around the cysts, to form the follicular epithelium, which then pinch off from the germarium to form a primary follicle (Koch *et al.*,1967). The follicle then develops according to the scheme proposed by Harlow (1956) (see general methods section), with the nurse cells developing polyploidy ( $n=512$ ) (King and Burnett,1959). These cells are rich in RNA which, it is proposed, may flow into the oocytes through ring canals before and during early vitellogenesis. The function of this RNA is not fully understood, but may be connected with the expression of maternal influences upon the developing eggs. This stage of development is reviewed by Engelmann (1970) and by King and Buning (1985).

In mosquitos, during vitellogenesis, the follicles gradually fill with a proteinaceous yolk also containing significant quantities of lipid and carbohydrate. The bulk of this yolk comes from protein precursors called vitellogenins which are produced from the adult fat body (Hagedorn *et al.*,1975) in response to the production of ecdysone by the ovary. The production of this ovarian ecdysone is initiated by egg development neurosecretory hormone, EDNH (Hanaoka and Hagedorn,1980) from the medial neurosecretory cells (Lea,1972). This neurosecretion appears to be triggered by a protein meal (Hagedorn and Fallon,1973). Little information is available on the involvement of EDNH in the flesh flies. A review of the process of vitellogenesis can be found in Kunkel and Nordin (1985).

The requirement for a protein meal for EDNH release and subsequent vitellogenesis in the flesh fly raises the question of autogeny, *i.e.*, the ability to undergo vitellogenesis in the absence of a protein meal. The ovaries of *Sarcophaga* are in a rudimentary state of development at eclosion. Some initial

ovarian development can take place in flies raised only on sugar and water, but this is small compared to the potential development following a protein meal. Most workers in this field have provided diets which promote optimum ovarian development and have therefore not concentrated on this question (Harlow, 1956; Sang and King, 1961; Morrison and Davies, 1964). Pappas and Fraenkel (1977), however, determined that *Phormia regina* when fed on sugar and water alone, deposited no yolk, but that *Sarcophaga bullata* under the same conditions initiated the deposition of yolk (incipient autogeny). They further determined that the quality of the protein meal was important. Purified protein (casein or gelatin) led only to initial yolk deposition in *P. regina* and had no additive effect beyond incipient autogeny to development in *S. bullata*. Full development required a protein source plus a vitamin and potassium salt mixture, as would be found in a meat or a blood meal. These workers suggested that full or incipient autogeny varies considerably with species, strain, larval rearing and adult condition. It is probable that this variability has led to the seemingly contradictory nature of the literature. Pappas and Fraenkel's (1977) conclusions agree with Denlinger's (1971) which showed that *S. bullata* only displays incipient autogeny. Baxter *et al.* (1973), however, regularly obtained full autogeny in larger individuals in this species. It would appear therefore that the quality and quantity of the larval diet can affect subsequent adult autogeny. The inclusion of cholesterol for example, into a sugar/water diet achieved 40–60% autogeny in the housefly *Musca domestica* (Robbins and Shortino, 1962), and Baxter *et al.* (1973) demonstrated that high feeding densities of *S. bullata* larvae led to small anautogenous flies. Spradbery and Schneizer (1981) also found that adult size influenced the expression of autogeny in the screw worm *Chrysomya bezziana*, small flies again being anautogenous.

The expression of autogeny in *S. argyrostoma* but not in *S. bullata* was briefly demonstrated by Denlinger (1971). Kenny (1985) concluded that: adult size was dependent on the length of the larval feeding period, the number of ovarian follicles correlated positively with the size of the adult, and that the expression of autogeny within these follicles was dependent upon adult size. He suggests that this size dependency was based on the quantity of reserves amassed during larval feeding. It was demonstrated that anautogenous vitellogenesis could begin in *S. argyrostoma* from the end of day 2 post eclosion with the introduction of the protein meal, but could be delayed until day 8. Most workers in this field have supplied a protein meal on day 4 (Denlinger, 1972; Wentworth and Roberts, 1984). Presumably therefore, the withholding of the meal until day 4 in anautogenous cultures can be used as a means of synchronising ovarian development.

Photoperiod and temperature are the environmental factors most responsible for facultative diapause induction (review in Saunders, 1982). The inductive or sensitive period to these factors does not however persist throughout the whole life cycle of a particular insect. Indeed, different periods of the life cycle are sensitive in different species. The sensitive stage and diapause stage are often temporally removed, although this is not always the case. Gibbs (1975) proposed that during the sensitive stage of *Sarcophaga argyrostoma*, photoperiodic information pertaining to diapause induction was stored in the form of a 'diapause titre'. This titre would be compared with an internal threshold at the end of the sensitive period, and diapause or development induced as appropriate.

Egg diapause in the mosquito *Aedes triseriatus* is directly induced by photoperiod experienced by the eggs (Clay and Vernard, 1972). In the linden

bug *Pyrrhocoris apterus* and in the Colorado beetle *Leptinotarsa decemlineata*, imaginal diapause is induced by short day photoperiods experienced by the young adults, although conditions during the nymphal or larval stages respectively can modify this response (Hodek,1971). In *Leptinotarsa*, short days can induce diapause in long lived adults which have already reproduced, the insects then overwintering for a second time (Ushatinshaya,1961). This degree of modification can also be seen in the red locust *Nomadacris septemfasciata* where imaginal diapause is induced by short days experienced as adults, but can be influenced by photoperiod in the nymphal stage (Norris,1965).

The sensitive stage in some insects immediately preceeds the diapause stage. In the early larval instars of the oriental fruit moth *Grapholitha molesta* (Dickson,1949), and the European corn borer *Ostrinia nubilalis* (Bell and Adkisson,1964), short days induce diapause in the mature larvae. Likewise, in the later instars of the silkmoth *Antheraea pernyi* (Tanaka,1950), the tomato moth *Diataraxia oleracea* (Way and Hopkins,1950) and the tobacco hornworm *Manduca sexta* (Rabb,1966) short days induce diapause in the supervening pupal stage.

In some species, the sensitive stage and the resulting diapause stage may be separated by one or more intervening instars, stages, or may even be in separate generations. In *Sarcophaga crassipalpis*, pupal diapause will only occur if embryos in the last two days of intrauterine development experience short days. If the first two days of larval life are also so illuminated, diapause induction is further increased. However, if only the larval stage is given short days, no diapause results (Denlinger,1971). In other flesh flies, *S. argyrostoma* (Saunders,1971), *S. bullata* (Denlinger,1972) and *Tricholioproctia impatiens*

(Roberts and Warren,1975), diapause can be induced even if only the larval stage is given short days. In *S. argyrostoma*, photoperiodic sensitivity has been shown to decrease from a maximum during the late embryonic stage through to the mature feeding larvae and cease during the post-feeding stage (Bradley,1984).

The mosquito *Aedes triseriatus* can enter diapause at two stages of its life cycle; either as an egg, directly induced by short days experienced as early instar larva, or as a fourth instar larva following short days during the preceding larval stages (Clay and Vernard,1972). An even more extreme separation of the sensitive and diapause stages can be seen in species where they are separated by generation. In *Aedes atropalpus*, short days during the fourth instar larvae and subsequent maternal stages give rise to, diapausing eggs (Kappus and Vernard,1967; Anderson,1968). A similar response is seen in the Psocopteran *Peripsocus quadrifasciatus* where diapausing eggs are produced in response to short days during the later maternal nymphal stages and early adult life. However, in this case, long day photoperiods in the young adults can reverse the short day response.

Larval diapause in the blowflies *Calliphora vicina* (Vinogradova and Zinovjeva,1972), *Lucilia caesar* (Ring,1967) and *L. sericata* (Fraser and Smith,1963) is under maternal control. In *C. vicina*, this is only displayed when the larvae are reared at 15°C or less. Likewise, the larval diapause of the parasitic wasp *Nasonia vitripennis* is under maternal control (Saunders,1965). In the case of pupal diapause in the horn fly *Lyperosia irritans*, the photoperiods experienced by the adult flies and the response of the immature stages to low temperatures interact to induce diapause or development (Wright,1970).

The silkworm *Bombyx mori* probably displays the greatest separation

between the sensitive and diapause stages. Embryonic diapause in this species is induced during the late embryonic and early larval stages of the previous generation. Winter diapause is therefore induced by the long days and high temperatures experienced by the summer generation (Kogure, 1933).

The question of maternal determination of diapause in flesh-flies was addressed by Denlinger (1971) who developed an artificial uterus to isolate developing eggs and embryos from *Sarcophaga crassipalpis*. This consisted of a petri dish containing moist filter paper onto which the eggs were squeezed from the female abdomens. He then covered the eggs with a cone of moist filter paper and sealed the dish with parafilm to preserve the humid uterus-like environment. The same rate of embryonic development was observed in the artificial system as in the intact females with no excessive mortality. He then transferred the isolated cultures from long to short-day photoperiods and *vice versa* on different days of embryonic development and noted that the diapause profiles were identical to those from naturally deposited larvae from intact females thereby concluding that there was no direct maternal influence in this species.

Working with *S. bullata*, Henrich and Denlinger (1982a) noted that the progeny from females with a diapause history could not be induced to enter diapause themselves by short day photoperiods at 20°C whereas those with no diapause history produced progeny with 40–70% diapause under the same conditions. Rockey and Denlinger (1986) later showed that the age of females with diapause history also affected subsequent diapause incidence; older females produced more diapausing individuals than younger females. Using the artificial uterus technique to isolate the eggs, Henrich and Denlinger (1982a) determined that this maternal influence was exerted prior to ovulation since

photoperiodic changes after this event could not induce diapause. Preliminary investigations by the same authors with *S. crassipalpis* indicated that a similar but much weaker maternal influence may be exerted on the developing eggs. A similar study by Bradley (1984) showed that no such mechanism exists in *S. argyrostoma*.

The second half of this chapter concerns itself with the post-embryonic development of *Sarcophaga* under diapause and non-diapause inducing conditions. Three strains of *S. argyrostoma* will be used in this portion of the investigation, namely STOCK, FAST and SLOW. These latter two were developed in Edinburgh by selecting from STOCK, over thirteen generations, those individuals which formed puparia early (FAST) or late (SLOW) in the larval wandering period (Bradley and Saunders, 1985). These workers found that two populations were established in which the pupariation profiles (measured with time) were discrete from each other. In continuous light at 25°C, the FAST strain pupariated about 3 days earlier than the SLOW strain. After communication with the present author, they suggested that no other aspect of development was altered. Henrich and Denlinger (1982b) also selected a strain of late pupariating flesh-flies (*S. bullata*) but determined that the selection in this species also increased the length of other, though unspecified, stages of the larval life cycle. These workers selected their late strain for six generations which delayed the mean of puparium formation by about 20h at LD 15:9, 25°C. At LD 12:12, 20°C however, the mean of pupariation was still only delayed by 24h, but the duration of the larval life cycle had increased from 6 to 22 days. They further demonstrated that the diapause incidence in the selected strain was far greater than that in the unselected stock strain. Bradley and Saunders (1985) however, showed that the diapause incidence in the FAST and SLOW strains was lower than that in



STOCK. They interpreted this in terms of a modified version of the photoperiodic 'counter' mechanism hypothesis proposed by Gibbs (1975) which has already been mentioned in this introduction. They suggested that the FAST strain which pupariated early experienced fewer diapause inductive short days than STOCK, and therefore accumulated a lower 'diapause titre' leading to the lower diapause incidence. When the wandering period was protracted, as in SLOW, they suggested that the 'diapause titre' dropped as a result of it being in some way unstable, to the extent that decay outweighed synthesis. The delaying of pupariation in STOCK larvae by overcrowding (Giebultowicz, unpublished observations, cited in Saunders and Bradley,(1984)), or by wet treatment (Ohtaki *et al.*,1968) also lowered diapause incidence (Saunders and Bradley,1984). This supported the Bradley and Saunders (1985) modified Gibbs hypothesis since any direct effects of strain selection could be ignored in these examples.

The effects of three of the factors introduced here (strain, photoperiod, and post-feeding crowding) on the post embryonic development of *S. argyrostoma* will be examined in this chapter. Any effects on the rate of larval growth, and on the length of the wandering period and subsequent diapause incidence will be investigated as a prelude to further more detailed studies of diapause in this species in later chapters. Prior to that however, a study of photoperiodic effects on ovarian and embryonic development, and the existence or otherwise of a maternal inductive influence will be presented.

### **Ovarian and embryonic development in long and short days**

Four larval cultures of approximately 400 larvae each were established in LD 18:6 and LD 12:12 at 25°C from 'FAST' strain adults reared in LL at 25°C as described in the general methods section. These cultures were set up such that half at each photoperiod experienced dawn at 09-00 (local time) and the other half at 21-00 (local time). Post feeding larvae were allowed to pupariate in single 6x3.5cm tubes to synchronise development. This will be discussed later in this chapter. Those which had pupariated within 48 hours were maintained at their respective photoperiods until eclosion, the remainder were discarded.

Adult flies were collected from the first major eclosion gate which occurred around dawn 12 days after pupariation. Meat for the protein meal was supplied 4 days after eclosion for 24h. Five adult females were collected every 6h from the appropriate photoperiod and their ovaries removed with forceps for follicle examination (Day 0-7). The data from the 09-00 and 21-00 (local times) dawn cultures were matched together to give a continuous set of measurements. Following ovulation, eggs or embryos were gently squeezed from the abdomens with forceps and examined. Ten follicles or eggs from each female were measured and the stage of development determined. The stages correspond with those proposed by Pappas and Fraenkel (1977) as described in the general methods section. An average size was calculated from the results from all five females.

Figure 1.1 shows the growth and development of the follicles from adult eclosion to ovulation in both photoperiods. In both cultures, growth can be divided into two sections; that prior to the protein meal and that after it.

Growth was slow until after the meal and in both the long and short day photoperiods, no differences in the rates of growth were noted. The follicles developed to stage 4a (0 to 1/4 full of yolk) in the absence of the protein meal. Whilst the follicles appeared to continue to grow in size slightly, no further yolk deposition was noted until 12h after the introduction of the protein meal. The timings of the developmental stages outlined in the general methods section are shown in table 1.1. It can be seen that there are few differences in the rates of development between the two lines in the follicular stages except that those follicles in LD 18:6 achieve maturity (stage 5) 12h earlier than those in LD 12:12. In the post- ovulation stages, it appears that the LD 18:6 line continued to develop more quickly than the LD 12:12 line. Figure 1.2 shows the proportion of embryos at each developmental stage after ovulation. In each case, (the presence of eggs in the uterus, the appearance of trachea and spiracles, and the initiation of larval movement within the chorion) the long day line developed faster by about 12h. Larviposition in the long day line occurred 20h earlier when allowed to proceed naturally. The variation in the proportion in each line with time is a product of the sampling method and an indication of the variability of developmental rates in this species.

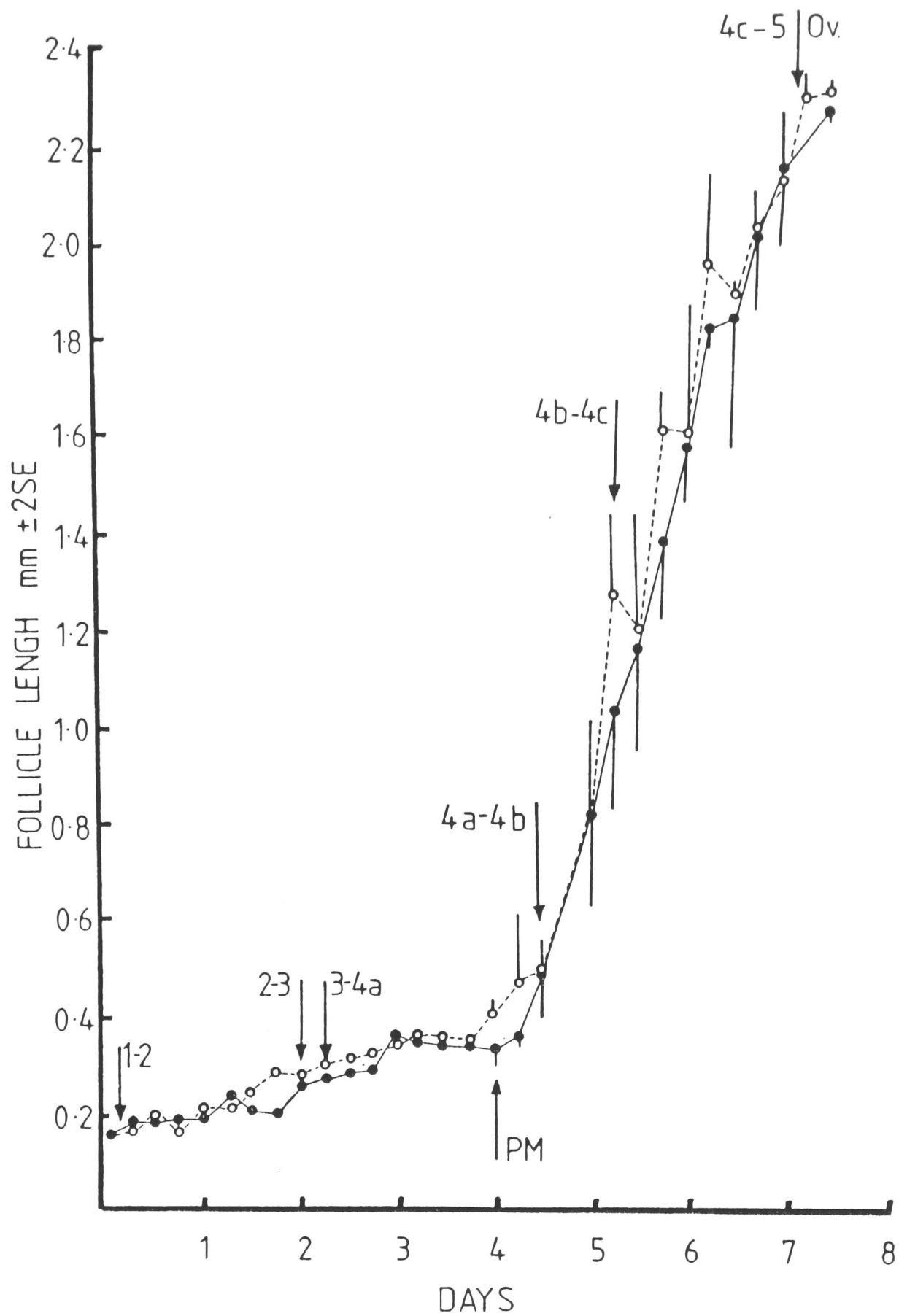


Figure 1.1: The growth and development of *Sarcophaga argyrostoma* ovarian follicles from adult eclosion *in vivo* under LD 18:6 (○-○) and LD 12:12 (●-●) photoperiods at 25°C. PM-Protein meal; Ov-ovulation. See text for details of stages 1 to 5. The timing of developmental events, indicated by arrows, shown in this figure are approximate. See Table 1.1 for times. Each point represents the mean of 10 determinations from each of 5 females  $\pm$  2SE.

Table 1.1 The timing (hours) of ovarian and embryonic stages of development of *Sarcophaga argyrostoma* in long and short day photoperiods. See the general methods section for details of each stage.

Ovarian stage	LD12:12	LD18:6
1	0-6	0-6
2	6-48	6-36
3	48-54	36-60
4a	54-108	60-102
4b	108-126	102-120
4c	126-170	120-156
5	170-180	156-180
Post ovarian stage		
Egg	180-252	168-240
Trachea	240-258	240-252
Spiracles	246-258	240-246
Movement	246-	246-
Larviposition	270	250

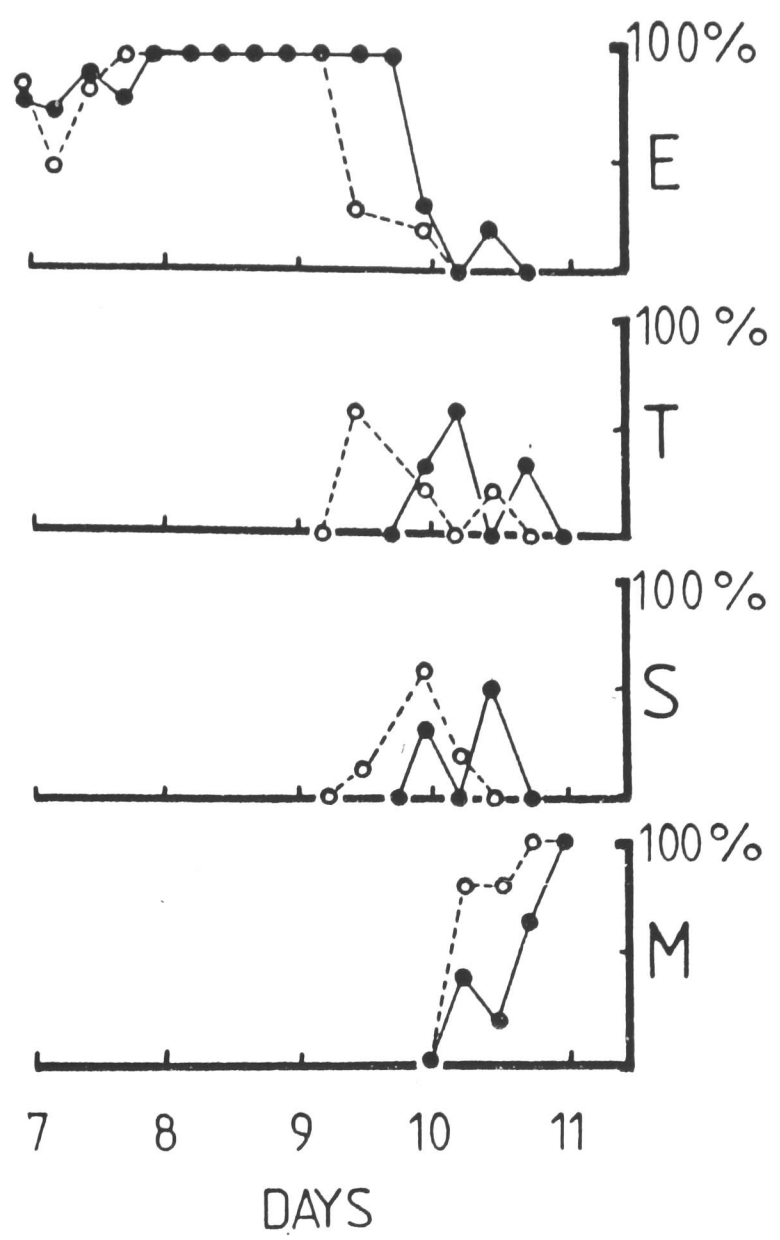


Figure 1.2: The development of *Sarcophaga argyrostoma* eggs and embryos after ovulation on day 7 post eclosion *in vivo* under LD 18:6 (○--○) and LD 12:12 (●--●) photoperiods at 25°C. E-egg; T-appearance of tracheae; S-appearance of spiracles; M-initiation of movement. See table 1.1 for times.

**Ovarian, embryonic and larval sensitivity to diapause inducing photoperiods *in vitro***

**Materials and methods**

Artificial uteri were constructed from small plastic petri dishes (5cm internal diameter) containing two circular pieces of filter paper (Whatman No 1). The paper was soaked with Grace's medium containing 1% v/v antibiotic solution (Sigma antibiotic/antimycotic solution. 10,000 units penicillin, 10mg streptomycin and 25 $\mu$ g amphotericin B per ml).

Adult FAST strain *Sarcophaga argyrostoma* females were removed from each photoperiod 7 to 8 days after eclosion and their abdomens squeezed gently with forceps to expel the eggs onto the filter paper. The dish lid was replaced to avoid undue evaporation and the dishes stored under the appropriate photoperiod at 25°C. The dishes were examined daily and the paper kept moist throughout embryonic development. Five females were used for each treatment giving approximately 100–200 eggs per cultures.

Prior to ovulation at day 7–8 post eclosion at 25°C, all the follicles are contained within the ovaries. Only after ovulation was it possible to squeeze the eggs into the artificial uteri. They were maintained in these dishes until the larvae had hatched from the chorion (Day 11–12 at 25°C), when the entire dish was inverted onto a piece of beef muscle on the agar diet supplement. If the larvae were transferred individually, or if the filter paper were placed on the meat directly, the rate of mortality was very high. The larvae were allowed to undergo normal development from that point onwards.

## Results

Two groups of *Sarcophaga* raised under LL at 25°C were allowed to eclose into LD 18:6 or LD 12:12 at 25°C respectively and meat supplied on day 4. On day 8, five females were removed from the LD 18:6 cage and their eggs squeezed into an artificial uterus. The eggs were then maintained at LD 18:6 for the remainder of their development. The progeny from another five females were transferred to LD 12:12. Similar groups were established from adult females reared under LD 12:12.

Table 1.2 shows the incidence of diapause in the groups established. It can be seen that when the post-ovulation egg/embryonic stage was maintained under LD 12:12, a diapause incidence of 89% was noted, regardless of adult photoperiod. No diapausing individuals were found when the dishes were maintained at LD 18:6. The levels of diapause in cultures allowed to develop normally in the adult female abdomens under LD 18:6 and LD 12:12 were 2% and 87% respectively. The use of the artificial uterus therefore has no effect on diapause induction in this species.

When eggs from cultures raised in either photoperiod were transferred to DD for artificial uterine and larval development, a low incidence of diapause is noted (Table 1.3A and B). The follicular stages therefore appear not to be sensitive to photoperiod. In the absence of any photoperiodic information (Table 1.3C), approximately half of the individuals entered diapause at 25°C. When post ovulation eggs/embryos were exposed to photoperiods (from day 7–11 post eclosion), diapause or development were more firmly induced (Table 1.3D and E for 18:6, and H and I for 12:12). The exposure of the pre-ovulation eggs to the photoperiods had little effect on diapause or



development induction (Table 1.3F and G for 18:6, and J and K for 12:12). When cultures raised in DD were transferred to LD 18:6 in artificial uteri, and then to LD 12:12 as larvae (Table 1.3L), diapause incidence was zero. This also applied in Table 1.3M when the photoperiods were reversed indicating that development was more firmly induced than diapause, regardless of the stage at which the cultures were exposed to LD 18:6 during the sensitive stages. When cultures raised in LD 18:6 were transferred to LD 12:12 either in the artificial uterus or at the larval stage, with the other stage in DD, a high diapause was noted (Table 1.3N and O). In the reverse cultures (P and Q), no diapause was recorded. These results confirm those of Denlinger (1971) that pre-ovulation eggs are not photoperiodically sensitive.

Table 1.2: The incidence of diapause (% D)in cultures from adult *Sarcophaga argyrostoma* allowed to eclose into LD 18:6 and LD 12:12 pre-ovulation photoperiods (OV) at 25°C which were then maintained after ovulation in artificial uteri in LD 18:6 and LD 12:12 (AU). Subsequent larval cultures were raised in the appropriate post-ovulation photoperiod. The level of diapause in cultures allowed to larviposit normally is also shown.

OV	AU	N	% D
18:6	18:6	71	0
18:6	12:12	63	89
12:12	18:6	90	0
12:12	12:12	82	89
18:6 Normal larviposition		163	2
12:12 Normal larviposition		227	87

Table 1.3: The effect of photoperiod on diapause induction (%D) at different stages of the *Sarcophaga* reproductive and growth cycle *in vitro* at 25°C. OV refers to the the photoperiod that the eggs were maintained under in *in vivo* in the ovary; AU refers to the photoperiod after ovulation when the embryos were maintained in the artificial uteri; L refers to the larval photoperiod; N is the number of individuals within each group.

Culture	OV	AU	L	N	% D
A	18:6	DD	DD	70	24
B	12:12	DD	DD	78	27
C	DD	DD	DD	98	47
D	18:6	18:6	DD	117	2
E	DD	18:6	DD	158	0
F	DD	18:6	18:6	108	2
G	18:6	18:6	18:6	107	0
H	12:12	12:12	DD	107	95
I	DD	12:12	DD	96	82
J	DD	12:12	12:12	57	88
K	12:12	12:12	12:12	212	86
L	DD	18:6	12:12	106	0
M	DD	12:12	18:6	130	1
N	18:6	DD	12:12	94	85
O	18:6	12:12	DD	36	95
P	12:12	DD	18:6	63	0
Q	12:12	18:6	DD	187	0

## **Larval development and puparium formation in long and short day photoperiods.**

### **Materials and Methods**

Cultures of STOCK, FAST and SLOW strain adult *Sarcophaga argyrostoma* were allowed to eclose in cages at LD 18:6, 25°C, and LD 12:12, 25°C. Meat was provided on day 4 post-eclosion for 24h to allow normal embryonic development to commence. On day 12, meat was put in the cages for 2h for larviposition to take place thereby providing a large number of newly deposited larvae of a similar developmental age. Several larval cultures (approximately 200 in each) were established from each adult culture in the appropriate photoperiods at 25°C on the meat and supplementary agar/milk/yeast diet.

The middle of the 2h larviposition period was designated time zero, and all subsequent larval time points measured from this. At time zero and 24h, and at 12h intervals after this, five larvae were removed from each culture until the onset of pupariation and weighed. Similar, though more detailed larval development data involving both weight curves and the timing of larval moults was obtained for FAST strain larvae during the course of the haemolymph ecdysteroid analyses which will be discussed in chapter 3. To supplement the data obtained from STOCK and SLOW cultures in this chapter, this FAST data will be presented here, and then re-presented in chapter 3 for the sake of clarity.

Following the major gate of larval wandering from the food (see chapter 2), half of the larvae from each culture were transferred to single tubes (6x3.5cm)

containing 1ml of dry sawdust, and half were maintained in crowded conditions (approximately 100 larvae in a mousetray containing 1cm of clean, dry sawdust). The cultures were then examined every 12h for the formation of puparia, and each group of pupae then examined 8 days later for evidence of development or diapause. In each of the three strains therefore, there were cultures maintained singly or in crowded conditions in both LD 18:6 and LD 12:12, all at 25°C.

## Results

The growth of STOCK strain larvae in LD 18:6 and LD 12:12 is shown in figure 1.3. It can be seen that there is little difference in the rate of growth between the two photoperiods, both reach a maximum mean weight of approximately 250mg prior to wandering, followed by a weight loss of about 75mg before the onset of pupariation on day 6 post larviposition. Figure 1.4 presents similar data for SLOW strain larvae. The rate of weight loss after wandering is apparently lower than that in the STOCK strain, and the first puparia form on day 7. Figure 1.5 shows data obtained from a similar investigation into the growth and development of FAST strain larvae which forms a part of the haemolymph ecdysteroid titre determinations presented in chapter 3. This figure will be presented again in that chapter.

The rates of growth were similar in both the long and short-day cultures (fig. 1.5). Both cultures showed a thousand fold increase in weight over the 72h period following larviposition. The timing of the two larval/larval moults during the postembryonic development of this species was found to be the same in the two photoperiods. Subsequent post-feeding weight loss by the long day culture was more rapid than that of the short day culture, but following larval exodus, those larvae raised under LD 12:12 conditions wandered for 24h longer than those under LD 18:6 before the onset of pupariation. The two cultures reached the same mean larval weight by the onset of pupariation. The diapause incidences of these long and short day cultures were 0 and 89% respectively. These data are very similar to those from the STOCK and SLOW cultures raised under the same conditions. When the weight data from the first 48h of post larviposition development shown in

figure 1.5 are logarithmically treated, as in figure 1.6, it can be seen that this portion of growth is exponential. After 48h, this is no longer the case. Again, there appears to be no difference between the cultures raised under LD 18:6 and LD 12:12.

Only after the onset of larval wandering can significant differences between the strains and photoperiods be seen. Figure 1.7 shows the pupariation profiles of STOCK cultures under LD 18:6 and LD 12:12 in both crowded and uncrowded conditions. It can be seen that in long-day photoperiods, pupariation occurs 12 to 24h earlier in the uncrowded cultures (day 6 vs day 7), whereas, in short-day photoperiods, this difference is greatly enlarged. The uncrowded larvae begin pupariation at the same time as the crowded larvae but it only lasts 3.5 days. In the group culture, it lasts 10.5 days. Both single and group cultures achieve the same degree of diapause incidence as each other. Those which pupariate early have a lower diapause incidence than those which pupariate towards the end of the profile. In long-day cultures, the diapause incidence is zero in both single and crowded conditions.

The SLOW cultures raised in the long-day photoperiod both began pupariation on day 6.5, and again, those maintained in uncrowded conditions, finish pupariation earlier than those which were crowded after feeding (figure 1.8). Diapause incidence in both conditions was zero. Under LD 12:12 however, the uncrowded larvae began pupariation on day 7 and carried on until day 16.5. The crowded cultures did not begin until day 9, and carried on until day 22.5, a considerably lengthened period. The diapause incidence of the uncrowded larvae was low in the early pupariaters, rising rapidly as pupariation was delayed. In the crowded larvae, this was even more extreme with a low

diapause incidence in those larvae which pupariated before day 14 and a high incidence in those after day 15. No drop in diapause incidence was noted in the very late pupariaters.

Figure 1.9 shows the pupariation and diapause incidence profiles for 'FAST' strain cultures raised under long/short day, crowded/uncrowded conditions. In a long-day photoperiod, pupariation is almost over by day 7 in the uncrowded culture, and by day 8 in the crowded one. This was about 24h quicker than the equivalent STOCK cultures. Diapause incidence was again zero. The short-day uncrowded culture did not differ much from the STOCK culture, but the crowded culture finished pupariation about 4 days earlier. Diapause incidence was again low in the early pupariaters, and high in the late ones.

When the overall diapause incidences of the three strains maintained under uncrowded conditions at LD 12:12 are examined irrespective of pupariation times, as in table 1.4, no differences in incidences are seen. In the group cultures however, both FAST and SLOW display a lower incidence than, both their equivalent uncrowded cultures and the STOCK group culture. Diapause incidence in the FAST strain fell by about 50% and that in SLOW by about 15%. The crowded STOCK culture incidence actually rose above the uncrowded level.



Table 1.4: The diapause incidence of STOCK, FAST and SLOW strains of *Sarcophaga argyrostoma* raised under LD 12:12 at 25°C under both crowded and uncrowded conditions.

Strain	Crowded	Uncrowded
STOCK	89.8%	79.0%
FAST	41.8%	84.7%
SLOW	69.7%	81.7%

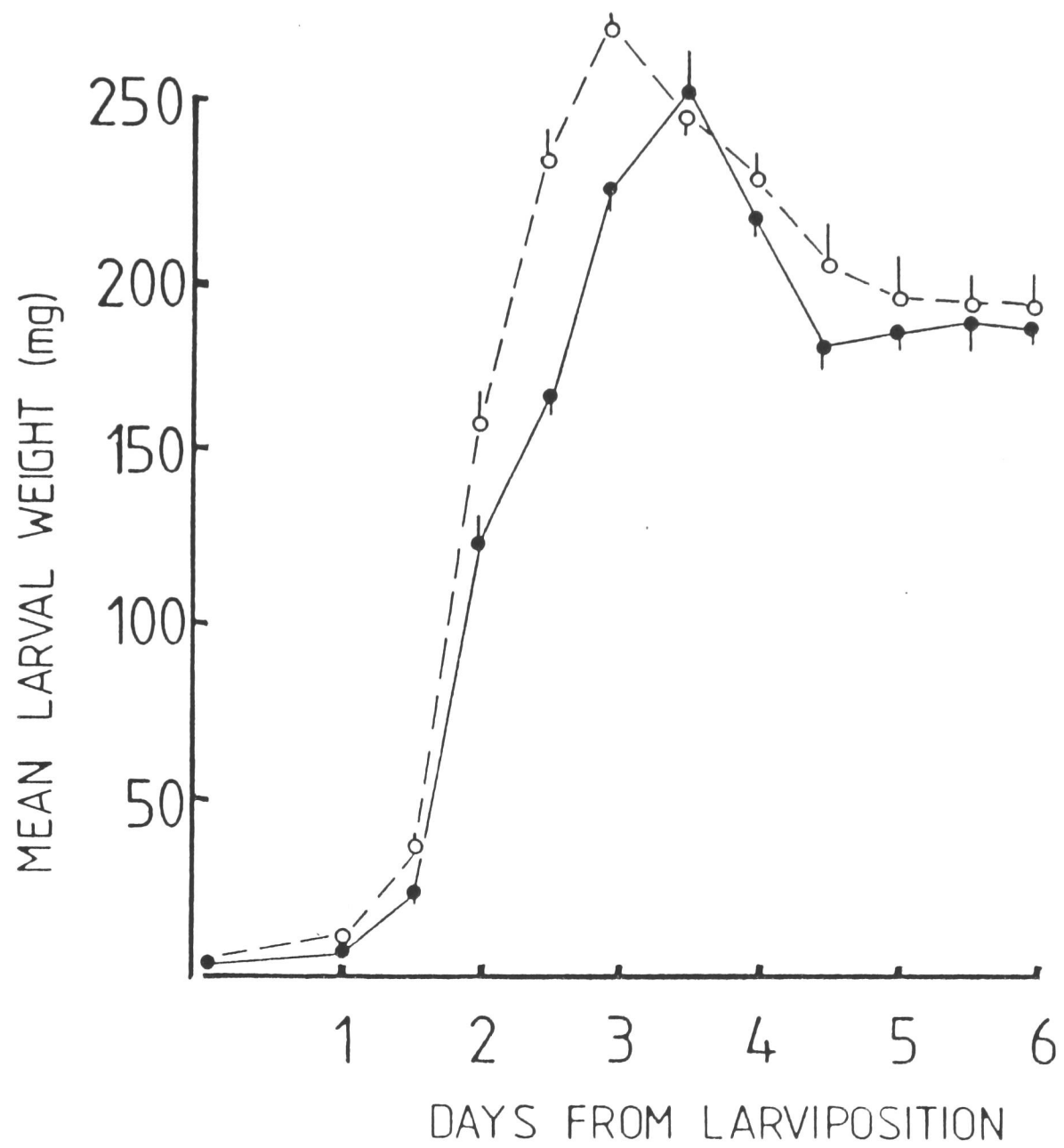


Figure 1.3. The growth of STOCK strain *Sarcophaga argyrostoma* larvae maintained at either LD 12:12 (●---●) or LD 18:6 (○---○) at 25°C. Each point represents the mean of five weighings  $\pm 1$ SE.

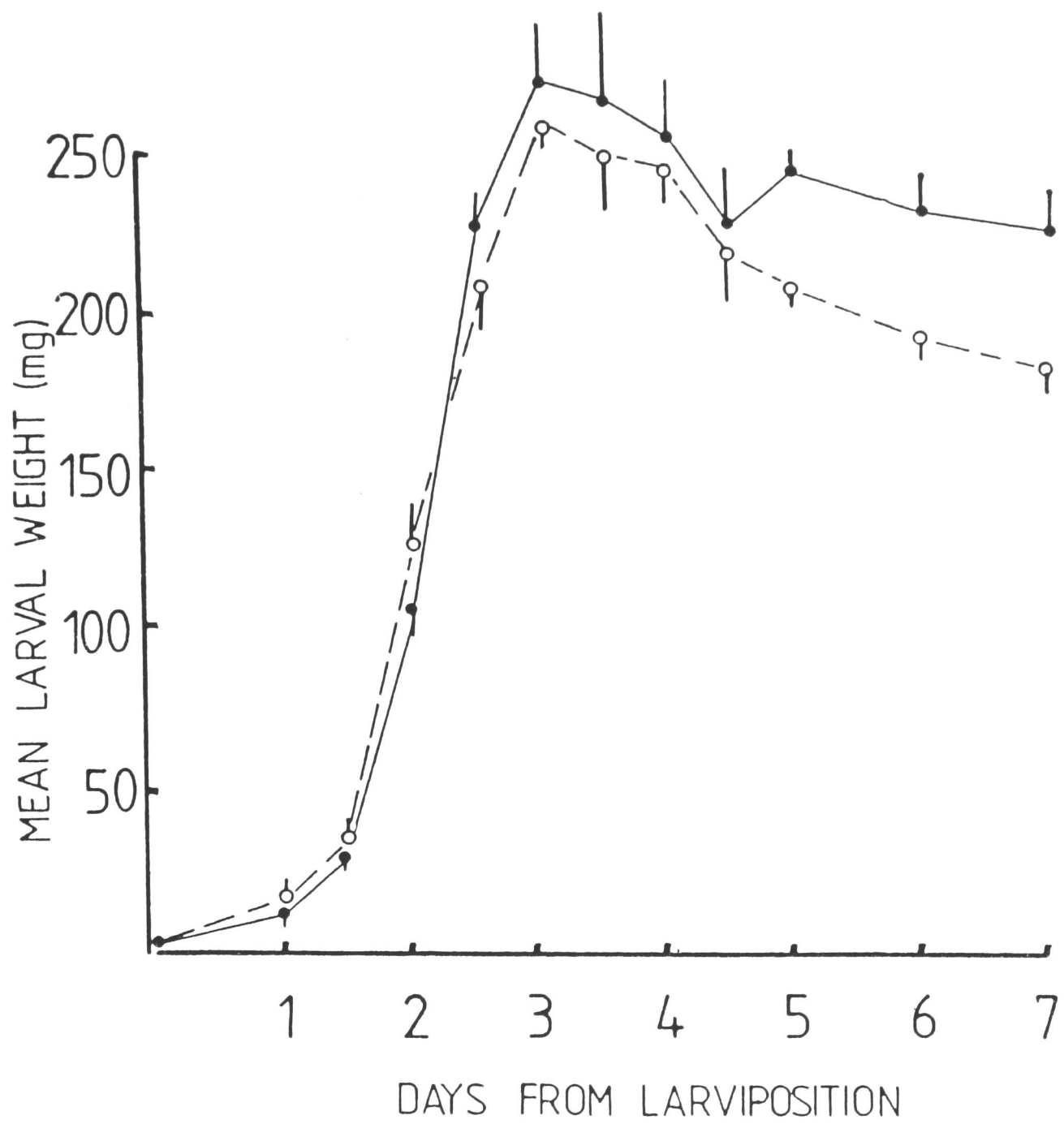


Figure 1.4. The growth of SLOW strain *Sarcophaga argyrostoma* larvae maintained at either LD 12:12 (●---●) or LD 18:6 (○---○) at 25°C. Each point represents the mean of five weighings  $\pm 1$ SE.

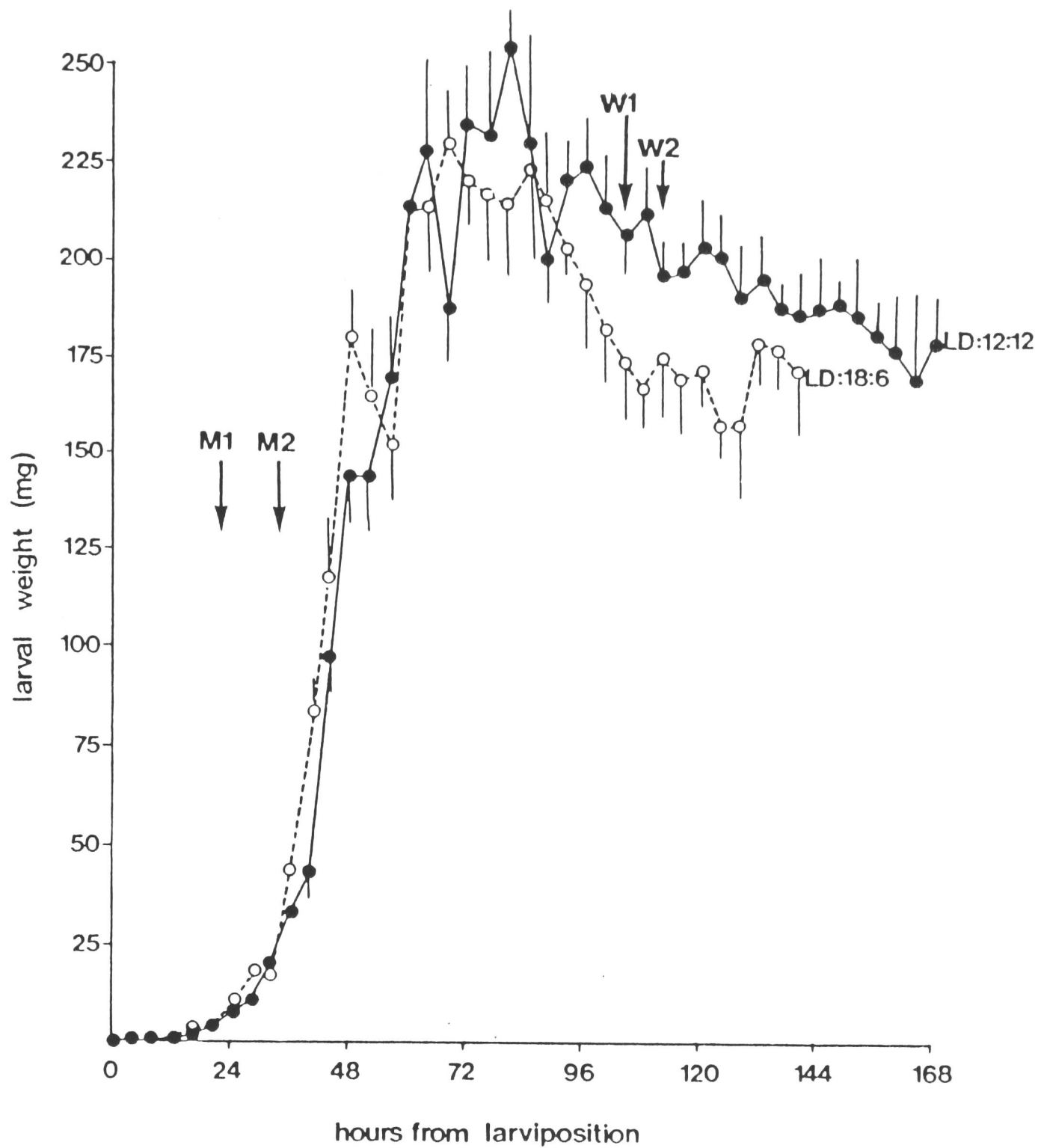


Figure 1.5. The growth and development of FAST strain *Sarcophaga argyrostoma* larvae maintained in either LD 12:12 (●---●) or LD 18:6 (○---○) conditions at 25°C. Each point represents the mean of five weighings  $\pm 2SE$ . M1 and M2 denote the first and second larval moults, respectively. W1 and W2 denote the onset of larval wandering from the food for LD 18:6 and LD 12:12, respectively.

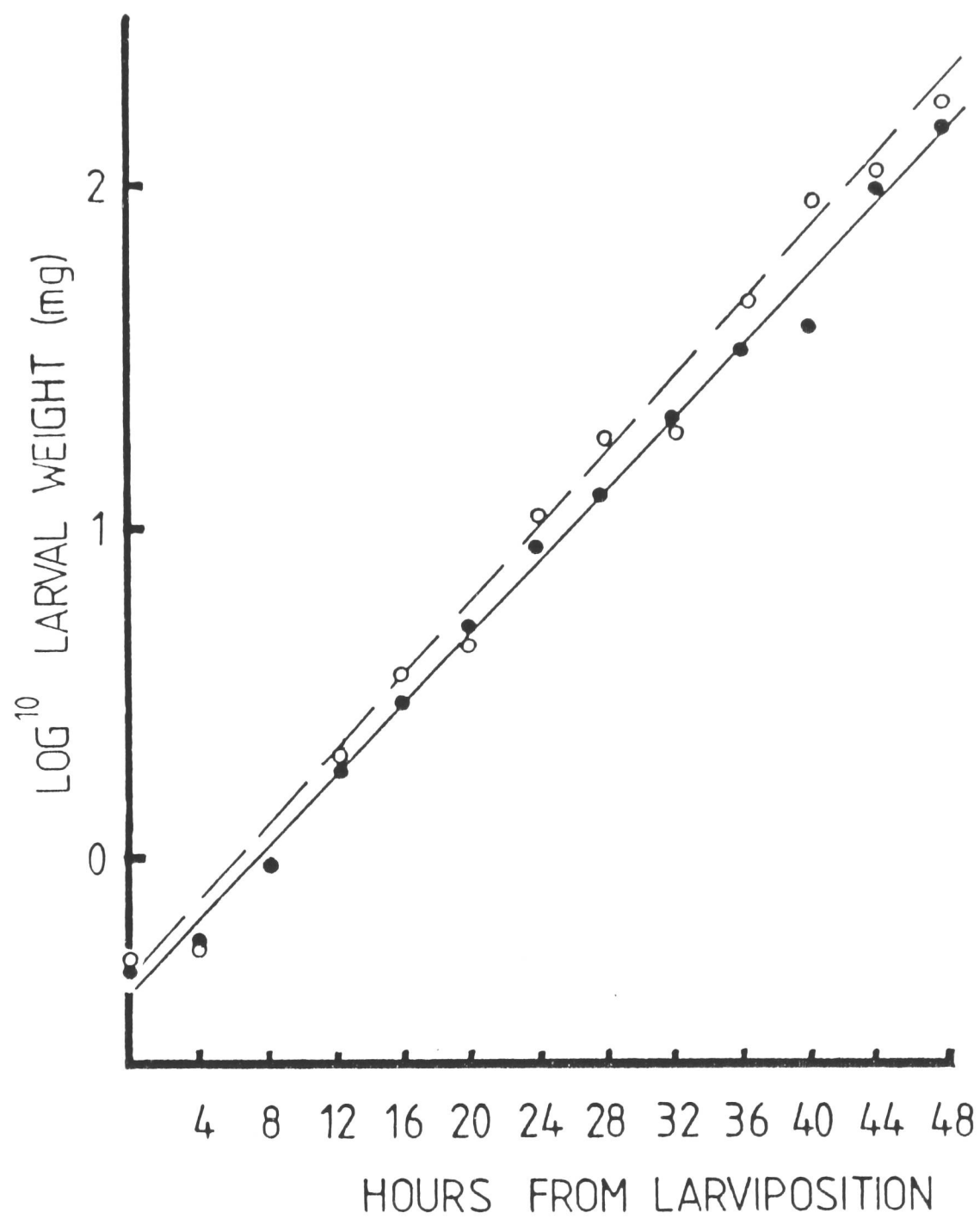


Figure 1.6. The growth rate of FAST strain *Sarcophaga argyrostoma* larvae maintained in either LD 12:12 (●---●) or LD 18:6 (○---○) at 25°C over the first 48h of post embryonic development showing that growth is exponential over this period. LD 12:12 correlation coefficient = 0.997, slope = 0.053, LD 18:6 correlation coefficient = 0.995, slope = 0.054.

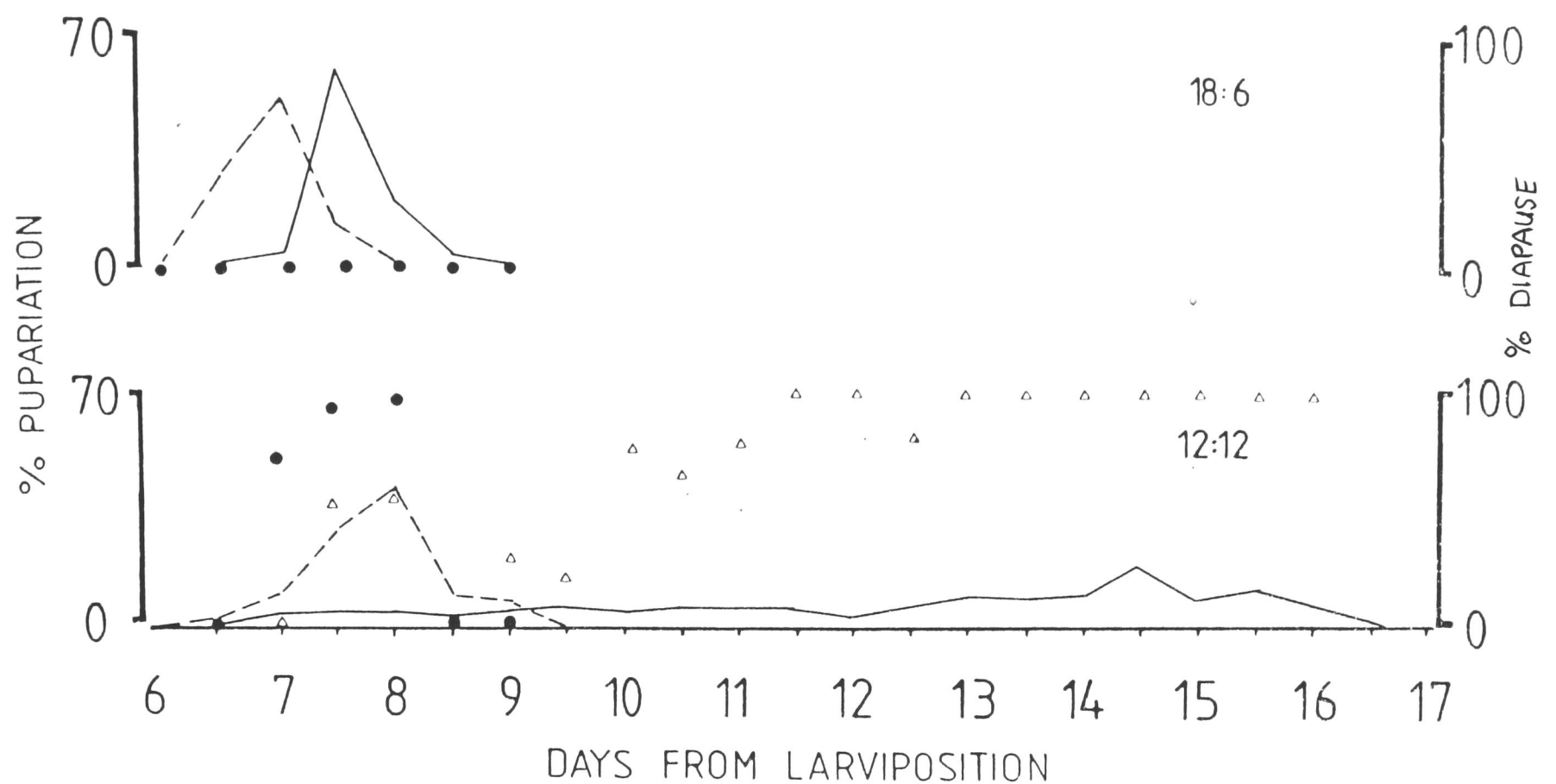


Figure 1.7. The pupariation profiles of STOCK strain *Sarcophaga argyrostoma* maintained in either LD 12:12 or LD 18:6 at 25°C. Larvae were kept in crowded (—) or uncrowded (----) conditions. The diapause incidence of each 12h collection group was determined 8 days after pupariation, crowded (Δ), uncrowded (●).

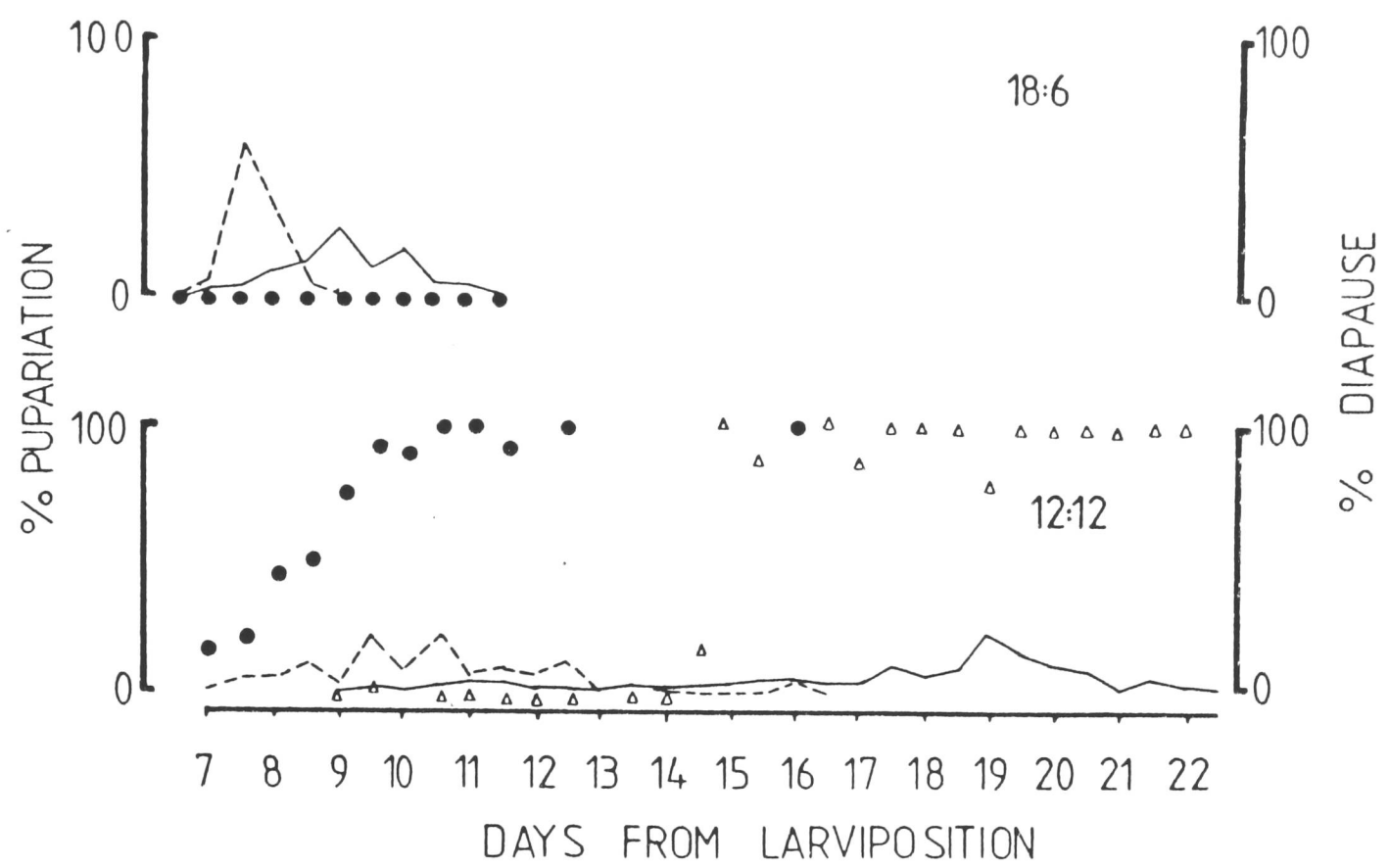


Figure 1.8. The pupariation profiles of SLOW strain *Sarcophaga argyrostoma* maintained in either LD 12:12 or LD 18:6 at 25°C. Larvae were kept in crowded (—) or uncrowded (----) conditions. The diapause incidence of each 12h collection group was determined 8 days after pupariation, crowded (△), uncrowded (●).

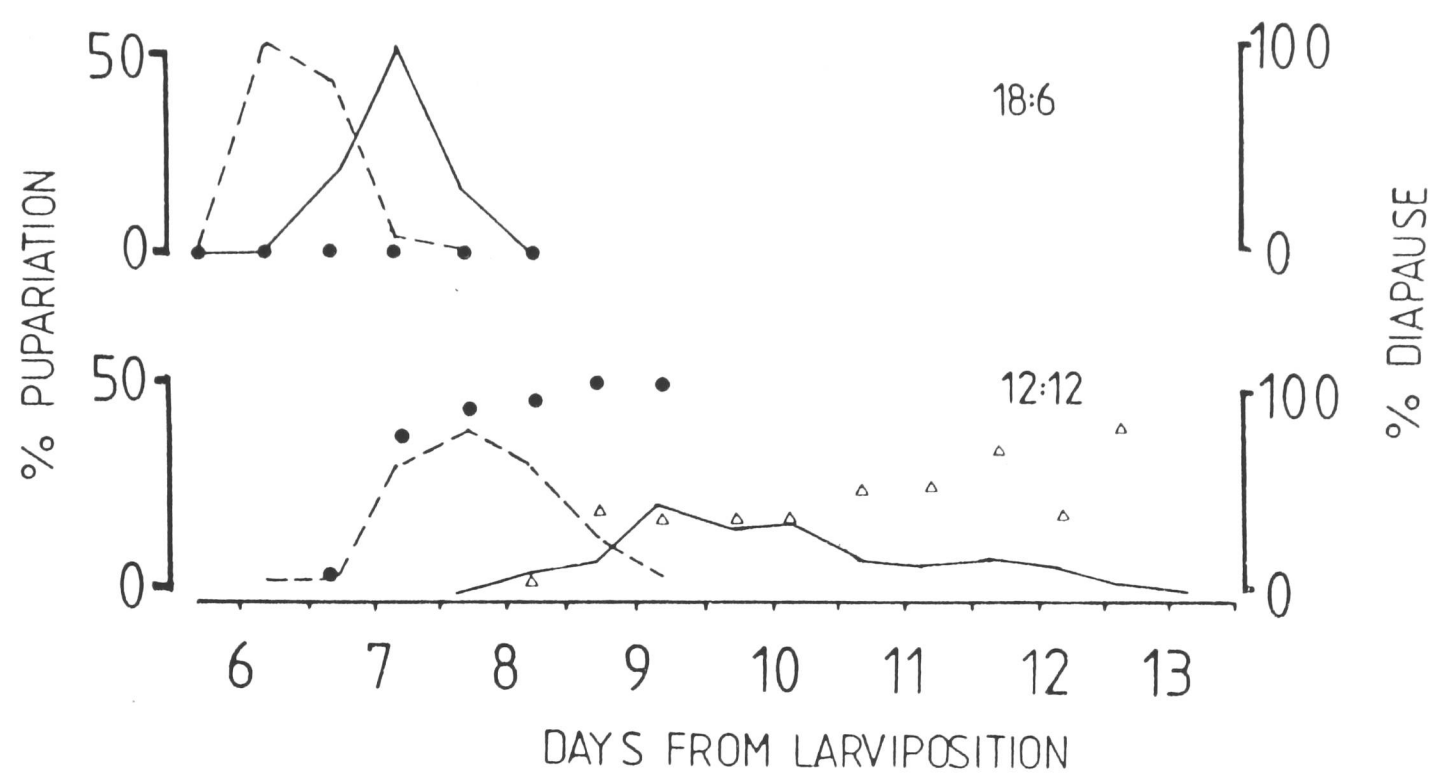


Figure 1.9. The pupariation profiles of FAST strain *Sarcophaga argyrostoma* maintained in either LD 12:12 or LD 18:6 at 25°C. Larvae were kept in crowded (—) or uncrowded (---) conditions. The diapause incidence of each 12h collection group was determined 8 days after pupariation, crowded (△), uncrowded (●).



## Discussion

The ovarian and uterine development of *Sarcophaga argyrostoma* (Edinburgh FAST strain) has been shown to progress according to a scheme originally proposed for *Protophormia terrae-novae* by Harlow (1956), and later modified by Pappas and Fraenkel (1977) to include *Phormia regina* and *S. bullata*. There appears to be little difference in ovarian development between *Sarcophaga* reared under LD 12:12 (diapause inducing) and LD 18:6 (non-diapause inducing) photoperiods. However, those flies raised under LD 18:6 progress through their developmental stages slightly quicker than those under LD 12:12. Despite efforts to standardise the temperatures within the light boxes, the extra six hours of illumination per day may have increased the temperature slightly thereby speeding up the growth rate. The flies in these investigations were raised under conditions designed to avoid the expression of complete autogeny, namely, under high larval feeding densities ( $\approx 400$ ) leading to adults not large enough to contain sufficient reserves for autogenous growth (Kenny, 1985). This allowed a degree of resynchronisation of developmental age with the introduction of the protein meal on day 4 after eclosion. In common with *S. bullata* (Denlinger, 1971), *S. argyrostoma* only displays incipient autogeny under these conditions, less than 25% of the follicle being filled with yolk (Pappas and Fraenkel, 1977). Kenny (1985) showed that this stage (4a) could be extended up to day 8 in anautogenous cultures, or truncated to day 2 by the timing of the introduction of the meat. Whilst non-diapause destined cultures passed through the stages of ovarian development slightly quicker, the follicles of both cultures attained the same



size before ovulation.

In the uterus, the non-diapause destined cultures continued to develop slightly faster than those reared under LD 12:12, again possibly due to temperature effects. Larviposition occurred approximately 20h earlier under LD 18:6 than under LD 12:12. Apart from these possible temperature effects, there did not appear to be any difference in development between the two lines. The significance of this lies with the observations of Bradley and Saunders (1985) who determined that the intrauterine stage in this species was maximally sensitive to photoperiodic diapause induction. Despite the fact that diapause or development is determined most strongly at this stage, the lack of differences implies a storage of information to be used later. Gibbs (1975) proposed such a storage of diapause inducing effects in the form of a 'diapause titre' which would be compared with an internal threshold at the end of the sensitive period. If this titre exceeded the internal threshold, the individual would enter pupal diapause; if not, the pupa would continue normal development. Bradley and Saunders (1985) modified this model to account for differences in the period of larval wandering and the consequent effect upon diapause incidence. This will be discussed later in this chapter in the context of data presented in the larval development section.

The question of maternal influence in diapause induction in this species was addressed in the artificial uterus experiments. It has been shown that the removal and culture of post-ovulation eggs from the females did not affect diapause induction. In no culture however, was diapause saturated (*i.e.* 100% incidence), probably due to the relatively high temperature (25°C) at which the experiments were conducted. Consequently any photoperiodically induced bias was clearly expressed in diapause incidence.

The lack of photoperiodic sensitivity prior to ovulation was shown by an equivalent low diapause in both LD 18:6 and LD 12:12 cultures, when transferred to constant darkness (DD) after ovulation. No maternal influence appears to have been exerted upon the eggs prior to this event. If it had been, one may have expected either or both cultures to express an appropriate response to the adult photoperiod. This agrees with Denlinger's (1971) conclusions based on his studies of maternal effects in *S. crassipalpis*. That maternal influences prior to ovulation occur in *Sarcophaga* spp. was shown by Henrich and Denlinger (1982) who demonstrated that the immediate progeny of *S. bullata* females which had a history of diapause could not be induced to enter diapause themselves by LD 12:12 at 20°C. Using an artificial uterus protocol, they demonstrated that the influence was exerted prior to ovulation, possibly during oogenesis. In the same study, a similar but weaker response was noted in *S. crassipalpis*. No such influence was found to exist in *S. argyrostoma* (Bradley, 1984).

Further confirmation of the lack of photoperiodic sensitivity in the ovarian stage was demonstrated by transferring cultures reared under LD 18:6 to LD 12:12 at either the artificial uterine or larval stage, with the other stage in DD. Both situations led to high diapause, the long day photoperiod having had little or no effect. In the reverse cultures, no diapause was noted.

When cultures were raised in DD from adult eclosion to puparium formation, approximately 50% of the individuals entered diapause. Since there was no opportunity for these cultures to respond to photoperiodic cues, diapause induction under these conditions presumably relies upon an inbuilt threshold within each individual. This threshold may be related to that proposed by Gibbs (1975) with an accumulation of diapause titre even in the

absence of a short day photoperiod. Those individuals which entered diapause may be assumed to be those with lower internal thresholds than those which continue development. Saunders (1971) showed that an increase in the larval breeding temperature produces a decrease in diapause incidence. At higher temperatures, development is faster and the larvae will be subjected to fewer diapause inductive cycles before the end of the sensitive period than at lower temperatures. Consequently, less diapause titre can be accumulated at higher temperatures, and a higher proportion of individuals continue with uninterrupted development. In addition, Gibbs (1975) suggested that the diapause titre may be unstable and decay after the end of the sensitive period. This decay may be accelerated at higher temperatures possibly compounding the lowering of diapause incidence reported by Saunders (1971).

That the embryonic stage is sensitive to photoperiodic influence was shown with both diapause and development being firmly induced when the embryos were illuminated. It is of interest to note that at this temperature, cultures subjected to LD 18:6 during either uterine or larval development, and to LD 12:12 during the other stage did not enter diapause. This would imply that the diapause titre decayed more rapidly in LD 18:6 than in LD 12:12. When LD 18:6 was given first, a rapid initial decay of produced titre did not allow enough to be accumulated under the subsequent short days to exceed the internal threshold. In the reverse situation, that titre which had accumulated under LD 12:12 decayed below the threshold under the long day photoperiod, resulting in low diapause. This situation would indicate that development is induced more firmly than diapause. If the temperature were reduced however, the time course of development, and therefore the number of inductive cycles leading to the accumulation of 'diapause titre' would increase. At a lowered temperature, the rate of decay of the titre may decrease and as a result, more

individuals would have titre levels above the proposed threshold and the incidence of diapause may increase (Saunders,1971).

These conclusions can be considered in the context of the ecology of this species. The response of the embryos and larvae to constant darkness is not easy to explain since this is not a commonly found environmental condition. That the population appeared to be biased towards development rather than diapause may relate to the situation found in autumn. In the decreasing photoperiod, if the temperature is warm enough for development the population may delay the onset of diapause by developing for another generation. Since diapause is a metabolically expensive event, a delay in its onset would increase the survival and competency of individuals. However, this strategy must risk the population being caught out by a cold period of weather after the commitment to full adult development has been made. No studies of the life history of wild flesh flies have been made so conclusions on the relation of the photoperiodic response to actual developmental events in autumn are difficult to make.

It has been shown that the rate of larval development is essentially the same in the three strains used in this portion of the investigation (STOCK, SLOW and FAST). Whilst it is true that there are small differences, the overall trends shown here agree with those demonstrated by Wentworth *et al.* (1981) in *S. bullata*. Accurate timings of the moults are only available for the FAST strain; again, these agree with those reported by these workers. This aspect of larval development will be more fully discussed in chapter 3 where the FAST data will be re-presented in the context of haemolymph ecdysteroid titres. It would appear therefore that selection for an extended wandering period (Bradley and Saunders,1985) has not affected the duration and intensity of the

feeding period. However, Henrich and Denlinger (1982b) noted that the selection of a late pupariating strain of *S. bullata* also extended other stages of larval development. When cultures of *S. argyrostoma* from the same strains were reared in diapause and non-diapause inducing conditions, no apparent differences in the larval feeding period were noted. Indeed, the logarithmic treatment of the FAST data further suggests the independence of feeding rates from photoperiodic effects. That growth is exponential over the first 48h of larval development is an indication of the adaptation of the species to its apparently opportunistic breeding behaviour, in that it takes advantage of available food sources as rapidly as possible without regard at this stage to later developmental options.

The differences between the strains manifest themselves after larval wandering. The data presented here confirm the conclusions of Bradley and Saunders (1985) that the lengthening of the wandering period can be selected. In all conditions, FAST and SLOW differ from each other, and from the original STOCK strain. The effects of maintaining the wandering larvae singly in tubes or in crowded conditions are also apparent in all three strains. Consistently, the wandering period is extended by crowded conditions as originally proposed by Giebultowicz (unpublished observations, cited in Saunders and Bradley, 1984). The range of pupariation times varied from FAST larvae maintained under LD 18:6 in uncrowded conditions (90% pupariated within 36h), to SLOW larvae in LD 12:12 in crowded conditions (pupariation extended over 14 days). The effects of photoperiod are also apparent from the data presented here. Larvae reared in non-diapause inducing conditions formed puparia earlier than those in diapause inducing conditions. Whilst there was always an overlap of pupariation times, the bulk of the diapause destined cultures were pupariating later in all three strains under both crowded and

uncrowded conditions. The results of these experiments have been used to determine the conditions of larval rearing in the later investigations in this thesis. Chapter 2 which concerns the circadian nature of larval wandering behaviour and pupariation was conducted with STOCK cultures to accentuate the range of the wandering period to attempt to attempt to discern the presence of any circadian control on pupariation. Post-feeding larvae were maintained in single tubes to avoid the complicating effects of overcrowding on any endogenous rhythm. The range of pupariation times of SLOW larvae was considered too great for practical use. Chapters 3 and 4 which address the endocrinology of diapause induction both use FAST larvae to attempt to synchronize pupal age as far as possible and to allow the rapid conclusion of the investigations.

Bradley and Saunders (1985) demonstrated that diapause incidence in both FAST and SLOW cultures was lower than that of STOCK under certain conditions. They interpreted this in terms of a modified version of the Gibbs (1975) 'photoperiodic counter/ diapause titre' hypothesis. In the present investigation, STOCK cultures raised under LD 12:12 at 25°C in both crowded and uncrowded conditions displayed a diapause incidence that varied depending upon the time of pupariation. The early pupariaters in both densities have lower diapause incidences than the late pupariaters. This variation was also noted in the FAST and SLOW cultures. In terms of the 'diapause titre' theory, the early pupariaters would have experienced fewer short days and therefore, assuming photoperiodic sensitivity late into larval life, have accumulated less titre. The late pupariaters, having experienced more short days displayed a higher diapause incidence. The problem with this idea arises when the crowded and uncrowded conditions are considered, most notably in the SLOW cultures. Crowded SLOW larvae which pupariated early (between 9

and 14 days after larviposition) displayed a low diapause incidence and late pupariaters, a high incidence. However, uncrowded SLOW late pupariaters which had experienced the same number of short days as the early pupariating crowded larvae, displayed a high diapause incidence. Similar overlap of pupariation times with different resultant diapause incidences were also shown in STOCK and FAST cultures. If 'diapause titre' were accumulated in short days, one would have expected equal diapause incidences in same-age pupariaters regardless of density conditions. The cause of the effects of crowding on diapause incidence is not known but presumably, if the 'titre' theory is correct, must either directly reduce the 'titre', affect the rate of synthesis of 'diapause titre' or raise the internal threshold thereby increasing the number of short days required for diapause induction. Bradley and Saunders (1985) further suggested that a greatly protracted larval wandering period in SLOW cultures had the effect of lowering diapause incidence. The rationale for this suggestion was that the 'diapause titre' may be in some way unstable, and may decay back below the internal threshold when larval wandering was extended beyond a certain limit. The present data do not support this hypothesis since no decrease in diapause incidence was noted in the late pupariating groups within each strain. When the overall diapause incidences of the STOCK, FAST and SLOW cultures in the present investigation were examined however, it was seen that SLOW and FAST did have a lower diapause incidence than STOCK. These incidences were comparable to those obtained by Bradley and Saunders (1985). In the present investigation, the lower diapause incidence was accounted for by the early pupariaters, and not the late ones as predicted by these workers.

The interpretation of these results in an ecological context may rest with the proposal by Denlinger (1972) that those larvae which are diapause destined



may wander for longer, thereby increasing the probability of finding a suitable site for pupariation. Since a diapausing pupa will require a subterranean site for several months during a time of adverse environmental conditions, a more careful 'selection' may optimize survival. Those larvae which are not diapause destined within a short day culture must presumably develop as rapidly as possible to reduce the risk of being caught by winter before they have had the opportunity to breed, and their progeny have had the opportunity to develop and form diapausing pupae. That crowding has the effect of extending the length of the wandering period may be involved in ensuring a more effective dispersal of larvae before puparium formation. This effect is present even in the non-diapause destined cultures, but is less pronounced than in those which were raised in short-days, presumably because, in the latter, the site will be required for longer. It is more difficult to speculate upon the fact that no change in diapause incidence was noted in the cultures which were maintained in uncrowded conditions. Post wandering crowding presumably only occurs in circumstances where a large number of larvae are present. In this case, it may be to the advantage of the population to allow some of the individuals to undergo another full life cycle, in an attempt to minimize the length of the diapause period, whilst holding others back in case of bad weather. Diapause is a metabolically expensive event and any means of reducing its duration would result in stronger emergent adults in the spring. In uncrowded conditions, an individual would presumably receive no information regarding the size of the population and therefore enter diapause rather than risk another life cycle. In none of the cases discussed here was diapause saturated, so an increase in the numbers undergoing full development may not confer much advantage upon the population, whereas the risk to a possibly small population of decreasing diapause incidence may be great.

In summary, the work presented in this chapter has demonstrated that few developmental differences exist between cultures raised under diapause and non-diapause inducing conditions. Ovarian, embryonic and pre-wandering larval development is the same in both conditions, despite the fact the diapause is most strongly induced, without maternal influence, during the embryonic and larval stages. After the larvae have left their food, photoperiod and density both affect the length of the wandering period. Three strains of *Sarcophaga argyrostoma* were examined in this chapter for their suitability for use in the later investigations within this thesis. In addition, the conditions in which larvae can be raised was investigated with a view to optimizing conditions in later investigations.

## CHAPTER 2

The timing of larviposition, larval wandering and puparium formation in *Sarcophaga argyrostoma*

**The timing of larviposition, larval wandering and puparium  
formation in *Sarcophaga argyrostoma***

**Introduction**

Many physiological and behavioural events in the life cycles of insects may be regulated by biological clocks (Saunders,1982). Pittendrigh (1954) demonstrated that pupal eclosion, the emergence of adult flies from the puparia, in mixed-age populations of the fruit fly *Drosophila pseudoobscura* occurs as a daily or circadian rhythm. In light-dark (LD) cycles, eclosion occurs during an allowed period of time, or 'gate', close to dawn. Other events do not have this association with a particular phase of the photoperiod, presumably when such association confers no selective advantage (Pittendrigh,1958). This chapter will investigate the circadian nature of three population events in *Sarcophaga argyrostoma*, namely larviposition, larval wandering from the food, and puparium formation, and will discuss these events in terms of the potential selective advantage to the species. Before this investigation, a brief outline of behavioural and developmental events under circadian and photoperiodic control in insects in general will be provided.

The best known 'population' rhythm is that of pupal eclosion in *Drosophila* species which was first studied by Kalmus (1935) and Bunning (1935), and later extensively studied by Pittendrigh (1954,1960,1965,1966). Mixed-age populations of *Drosophila* raised in LD cycles emerge as adults in a well

defined rhythm with maximum numbers emerging close to dawn. Mixed-age populations are required to demonstrate such a rhythm because in any one individual, eclosion occurs only once. The peaks assume a close phase relationship with the LD cycles which entrain the rhythm (Pittendrigh, 1965), with the position of the peaks depending upon the length of the photoperiod. In short-day photoperiods, eclosion occurs before dawn whereas in long-day photoperiods, it occurs after dawn. In photoperiods longer than 18h, the phase relationship changes and in very long photoperiods or continuous light (LL), the rhythm breaks down.

One of the major requirements of a true circadian rhythm is that it should persist following the removal of the entraining agent (zeitgeber), for example a change in photoperiod from LD 12:12 to continuous darkness (DD). In these cases, the rhythm is said to 'free-run'. Pittendrigh (1954) demonstrated that this would occur with the pupal eclosion rhythm in *Drosophila*, the period remained close to 24h providing evidence for the action of an internal clock or endogenous oscillator controlling the rhythm. Were it under the control of the exogenous LD cycles, one would have expected the rhythm to collapse. In cultures which had received no photoperiodic or temperature cycles on which to entrain during development (*i.e.* those raised under LL or DD), no rhythmicity was apparent. However, a single step down from LL to DD was sufficient to initiate the rhythmic emergence of the adults. The introduction of a 12h 28°C temperature pulse to a population raised at 20°C under DD was also sufficient to induce the subsequent 24h rhythm with emergence maxima occurring close to the onset of the temperature rises, had they continued with a zeitgeber period (T) of 24h (Zimmerman, 1969). These observations suggest that circadian oscillators do not function until they are initiated by a light or temperature perturbation. An interesting observation by several authors (Bunning, 1935;

Pittendrigh,1954; Brett,1955) is that the oscillators in *Drosophila* can be set by these changes at any stage of larval or intrapuparial development . In the Queensland fruit-fly *Dacus tryoni* (Bateman,1955) and in *S. argyrostoma* (Saunders,1976,1979) however, entrainment and phase control cannot be induced during pupal stages. In these species, sensitivity ends at puparium formation, by which time they are subterranean and not exposed to light cycles. In *Dacus*, the eclosion rhythm can actually be set by the exposure of the previous generation to light/dark cycles indicating that embryos can 'inherit' circadian information from their parents.

A second prerequisite of a circadian rhythm is that the natural period ( $\tau$ ) of the rhythm be temperature compensated; *i.e.* that an increase in temperature should not change the length of the period. Pittendrigh (1954) again demonstrated that the eclosion rhythm occurs with a period of 24h at 26°C and 24.5h at 16°C indicating near total temperature compensation; the temperature coefficient  $Q_{10}$  was 1.07. Whilst the period of the peaks did not change, the phase relationship and the amplitude did, with fewer but larger peaks at the higher temperature.

Pupal eclosion rhythms have been described in a number of other species, for example, the giant silkmoths *Hyalophora cecropia* and *Antheraea pernyi* (Truman and Riddiford,1970). Both species emerge at specific times of the day. In *H. cecropia*, emergence occurs in a broad peak 1 to 9 hours after dawn, whereas in *A. pernyi*, it occurs later in the afternoon in long-day photoperiods and in the early part of the night in short-day photoperiods. When transferred from LD 17:7 to DD the *A. pernyi* rhythm free runs with a period of 22h (Truman,1971), which is shorter than that of *Drosophila*.

A second rhythm which has been extensively studied is that of oviposition.

Haddow and Gillet (1957) showed that oviposition in caged populations of the yellow fever mosquito *Aedes aegypti* occurred in well defined peaks towards the end of the light period in LD 12:12, a typical tropical photoperiod. As with the eclosion rhythm, the phase relationship was a function of the photoperiod. In short days, (LD 4:20), the peaks moved into the dark. Populations raised in DD showed weak rhythmicity, but with as little as 5 minutes of light per day, the distinct rhythm returned (Gillet *et al.*, 1959) with a period close to 24h. On transfer of a mixed-age population from either LD 12:12 or LL to DD, the rhythm persisted for at least eleven cycles. In the pink bollworm *Pectinophora gossypiella*, the oviposition peaks occurred during the early part of the night and continued for about 7h (Pittendrigh and Minis, 1964; Minis, 1965). However, in this case, the duration of the photoperiod had little effect on the phase relationship of the peaks to the light cycle. This was attributed to a complete suppression of oviposition activity during the light. When transferred to DD however, an endogenous periodicity of 22h 40 minutes was observed (Minis, 1965). Oviposition in *Drosophila* spp., which displayed such a pronounced eclosion rhythm did not appear to be strongly controlled by the circadian system (Saunders, 1982).

Larval rhythms have also been investigated, most notably the rhythm of larval wandering. Causse (1974) studied the 'larval jumping' behaviour rhythm of the fruit-fly *Ceratitis capitata* and noted that emergence from the artificial medium on which the larvae were raised occurred in a sharp peak (3 to 4h wide) close to dawn. In short-day photoperiods, the peaks phase lead dawn (*i.e.* occur in the late dark period) but in long-day photoperiods, they phase-lag dawn. The rhythm free runs with a period close to 24h and even persists in LL albeit with a broader peak width and a tendency toward arrhythmicity. Smith *et al.* (1981) demonstrated that larvae of the sheep blowfly

*Lucilia cuprina* leave their food at night, but the transfer of cultures from LD 12:12 to DD revealed only a very weak association with the circadian system. A similar association of larval wandering with the dark period was shown in the screw-worm fly *Chrysomya bezziana* (Spradbery *et al.*,1983). This may have been the result of exogenous stimulation rather than an endogenous rhythm since no free-run was attempted by these workers. Roberts (1984) noted that larvae of *S. bullata* also left their food during the night. This nocturnal peak of wandering was shown to coincide with a pulse of ecdysteroids in the haemolymph which was preceded by an increase in the level of ecdysone synthesized by the ring glands *in vitro*. This was presumably the response to an even earlier release of the prothoracicotropic hormone (PTTH) from the brain. In the tobacco hornworm *Manduca sexta*, this release of PTTH is a gated event (Truman and Riddiford,1974; Gilbert *et al.*,1981; Dominick and Truman,1984). Another larval rhythm which has been shown to exist is that of larval moulting. In the silworm *A. pernyi* and in *Manduca sexta*, ecdyses occur in sharp peaks. Truman (1972) demonstrated that the moults themselves were not controlled directly by the circadian system, but rather that the release of PTTH which initiates the synthesis of the moulting hormone ecdysone, was the gated event.

The rhythmic formation of puparia has been described in several Dipteran species, for example in the fruit fly *Drosophila victoria* (Rensing and Hardeland,1967). The salt marsh mosquito *Aedes taeniorhynchus* showed a faint sinusoidal rhythm of pupation with a period of 21.5h when raised from the egg stage in DD (Nayar,1967a,1967b). This rhythm was temperature compensated and could be made more pronounced by an unrepeated light pulse in the last larval instar, possibly as a result of previously random oscillators within the individuals of the population becoming synchronized.



Interestingly, the rhythm of adult eclosion in this species is not clock controlled, but merely reflects the antecedent rhythm of pupation (Neilsen and Haegar, 1954).

Saunders (1986) has reviewed the developmental and behavioural events in the life cycle of *Sarcophaga argyrostoma* which are under the control of circadian oscillators. Some of the rhythms he discusses are presented in this chapter as part of the current thesis. He suggests that the circadian system in this species contains at least three, and possibly six separate circadian pacemakers. This proposal will be discussed more fully after the presentation of the data in this chapter.

## Materials and Methods

Cultures of STOCK strain *S. argyrostoma* were raised as adults in cages under continuous light at 25°C as described in the general methods section. The flies were supplied with sugar and water *ad libitum*, and with a daily supply of fresh beef muscle. Newly deposited larvae were transferred on pieces of meat to the milk/yeast/agar supplementary diet. Fully fed larvae were allowed to wander into dry sawdust to form puparia.

In the experimental cultures, 200–300 first instar larvae deposited over a period of 2 or 24h were set up as above but exposed to a variety of light–dark (LD) cycles, or to continuous light (LL) or dark (DD) in light–tight boxes at 20°C. The precise conditions are described in the results section.

When wandering commenced, larvae falling into the sawdust were collected and counted hourly until all of them had left the food. Those larvae leaving the food during the dark period of the experiment were counted under a red safe light. The timing of larval wandering was in all cases non-random, usually occurring in a number of discrete peaks in both LD and DD regimes. Following Winfree (1970) in his study of pupal eclosion in *Drosophila pseudoobscura*, peaks of activity were defined as the densest 8h in each cycle. The distribution of larval exodus within and around each peak thus defined was frequently skewed.

In the experiments on puparium formation, newly wandering larvae were placed individually in 6x3.5cm plastic tubes containing 1ml of dry sawdust to avoid the complicating effects of overcrowding on the rate of pupariation (See

chapter 1). Every hour, newly formed (white or pale brown) puparia were recorded: the time of pupariation could then be related to both local time and to the time of wandering. A control group of larvae was manually extracted from the food before exodus began and then examined for periodicity of pupariation.

### **The timing of larval exodus in daily light cycles**

Seven cultures of larvae, deposited during a 2h period in constant light (LL), were set up in LD 12:12, 20°C with the light-dark transition occurring 4h later in each regime (Figure 2.1). Larval wandering commenced after 5 days and continued for about 36h with all cultures showing one or two discrete peaks of exodus. For the cultures A,C,D and E, the peaks occurred close to the onset of darkness, the peaks following the sequentially later transitions from light to dark. In cultures B,F and G, some larvae left the food during the light phase suggesting that they were able to follow a delay in lights-off only to a certain point before phase-jumping back to an earlier transition.

Figure 2.1H shows the same data summed according to photoperiod. This treatment produced an artificial or 'synthetic' mixed-age population, a technique proposed by Pittendrigh (1966), and clearly shows that larval exodus is rhythmic in an LD 12:12 cycle.

### **Larval exodus in light cycles with varying night lengths**

Seven cultures were established in differing light-dark cycles from adults maintained in LL with the lights-on transition occurring at the same local time (Figure 2.2,B-H). One culture was set up in constant darkness (DD) (Figure 2.2A) and one in LL (Figure 2.2I). The larvae were deposited over a 24h period to provide a mixed-age population resulting in a greater spread of developmental age, and consequently a greater number of activity peaks.

Most cultures produced larval exodus patterns with two or three clear peaks. In the longer night lengths (Figure 2.2,B and C) larval exodus occurred exclusively in the dark, but as night length shortened, and the LD transition

occurred later relative to larval age (Figure 2.2,D and E), the peaks showed a stronger association with the onset of darkness. Culture F showed a peak of wandering in the light as the larvae phase-jumped back to an earlier dark phase (Figure 2.2G). In the shortest night (Figure 2.2H), exodus commenced in the dark phase but continued into the next light phase. Wandering behaviour in DD (Figure 2.2A) following a transition from LL after larviposition, and in LL (Figure 2.2I) was arrhythmic. This demonstrates that in this species, a simple 'step-down' from LL to DD is not sufficient to induce this rhythm, although it is for pupal eclosion (Saunders,1976).

Figure 2.3 shows the phase relationship of larval wandering behaviour to the photoperiod. In photoperiods shorter than LD 9:15, the median of larval exodus occurred during the dark period. As the photoperiod increased from LD 9:15 to LD 18:6, the median of exodus followed the onset of darkness before, at LD 21:3, it was unable to maintain this close association, presumably because the dark period is too short.

#### **Transfer from LD to continuous darkness**

To investigate the possible circadian basis of the rhythmic initiation of wandering, mixed-age cultures of larvae were transferred to DD after two or three cycles of LD 12:12 with the final transfer occurring at different local times (Figure 2.4,A-G). Results shown in figures 2.1, 2.2 and 2.3 have already demonstrated the association of wandering with a continuous LD regime.

Cultures A,B,D and E showed two or three discrete peaks, clearly 'free-running' in DD with a circadian period of less than 24h. Cultures C,F and G, however, showed a breakdown of this pattern possibly as a result of a transient state (C) or the inability of the rhythm to persist in those cultures

which had spent the longest time in darkness (F and G). Since Figure 2.2A showed that a simple transfer from LL to DD was insufficient to initiate the circadian rhythm, it is understood that exposure to several LD cycles is required for the entrainment of the system, and that the rhythm, once initiated, does not persist for long in the absence of further time cues.

The circadian period ( $\tau$ ) of the rhythm in DD, calculated as the mean of the times between the medians of the peaks in the clearer 'free-runs' of Figure 2.4 (A,B,D and E) was approximately 21h.

#### **The timing of puparium formation**

Two mixed-age cultures of larvae were set up in the LD 12:12 photoperiod. In one (Figure 2.5A), the fully fed larvae were extracted manually from their food just before exodus commenced; in the other (Figure 2.5B), larvae were allowed to leave the food naturally. In both cultures, larvae were transferred to single tubes and puparium formation was recorded every hour.

Although larval exodus was clearly gated (Figure 2.5B) and largely nocturnal, puparium formation in both groups (A and B) was at random with no evidence of rhythmicity. Figure 2.5C also shows pupariation in B arranged according to the time in hours, from larval exodus, rather than according to larval age or the LD cycle; once again, there was no evidence of rhythmicity. The range of pupariation times in figure 2.5B (about 84h) was expected since the spread of larval exodus was about 60h. However, the enormous range of pupariation times (49–133h) observed when the data are plotted against time since exodus (Figure 2.5C), was surprising. The result illustrates that little, if any temporal control operates for puparium formation, in marked contrast to the stronger circadian regulation of larval exodus.

### **The timing of larviposition**

A culture of adult flies was allowed to eclose into LD 12:12 at 25°C and reared according to the general methods section. From day 12 post-eclosion to day 13.5, meat was placed in the cage and substituted every hour. The number of first instar larvae deposited each hour were counted. During the dark phase, all operations were carried out under a red safe light. Figure 2.6 shows that under these conditions, larvae are deposited at most times of the day or night with no evidence of periodicity. During the first 12h period,  $69 \pm 17$  (Mean  $\pm$  SEM) larvae were deposited per hour, during the dark period,  $79 \pm 17$ , and during the second day,  $50 \pm 10$ . After the second light period, larviposition rates were not measured every hour, but very few were seen to be deposited. The lower average during the second day may be a result of the females having exhausted the numbers of larvae to be deposited.

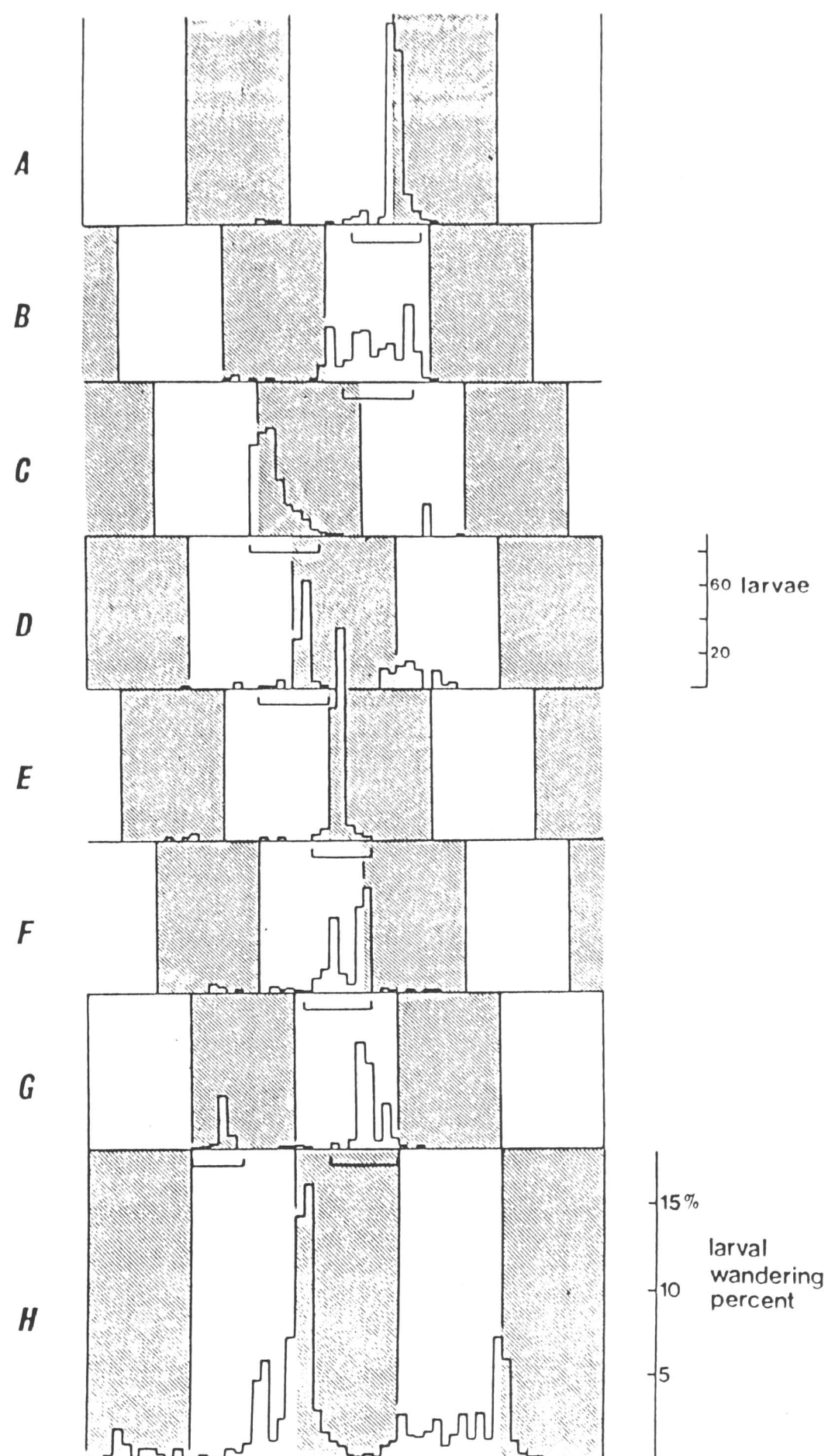


Figure 2.1. Larval wandering (exodus behaviour) in seven cultures (A- G) of the flesh fly *Sarcophaga argyrostoma*, maintained in LD 12:12, 20°C, with different phase relationships between the light cycle and larval age. The horizontal brackets mark the most dense 8h of activity in each recognised peak. H, data from A-G summed according to the light-dark cycle, giving a 'synthetic' mixed-age population (Pittendrigh, 1966).



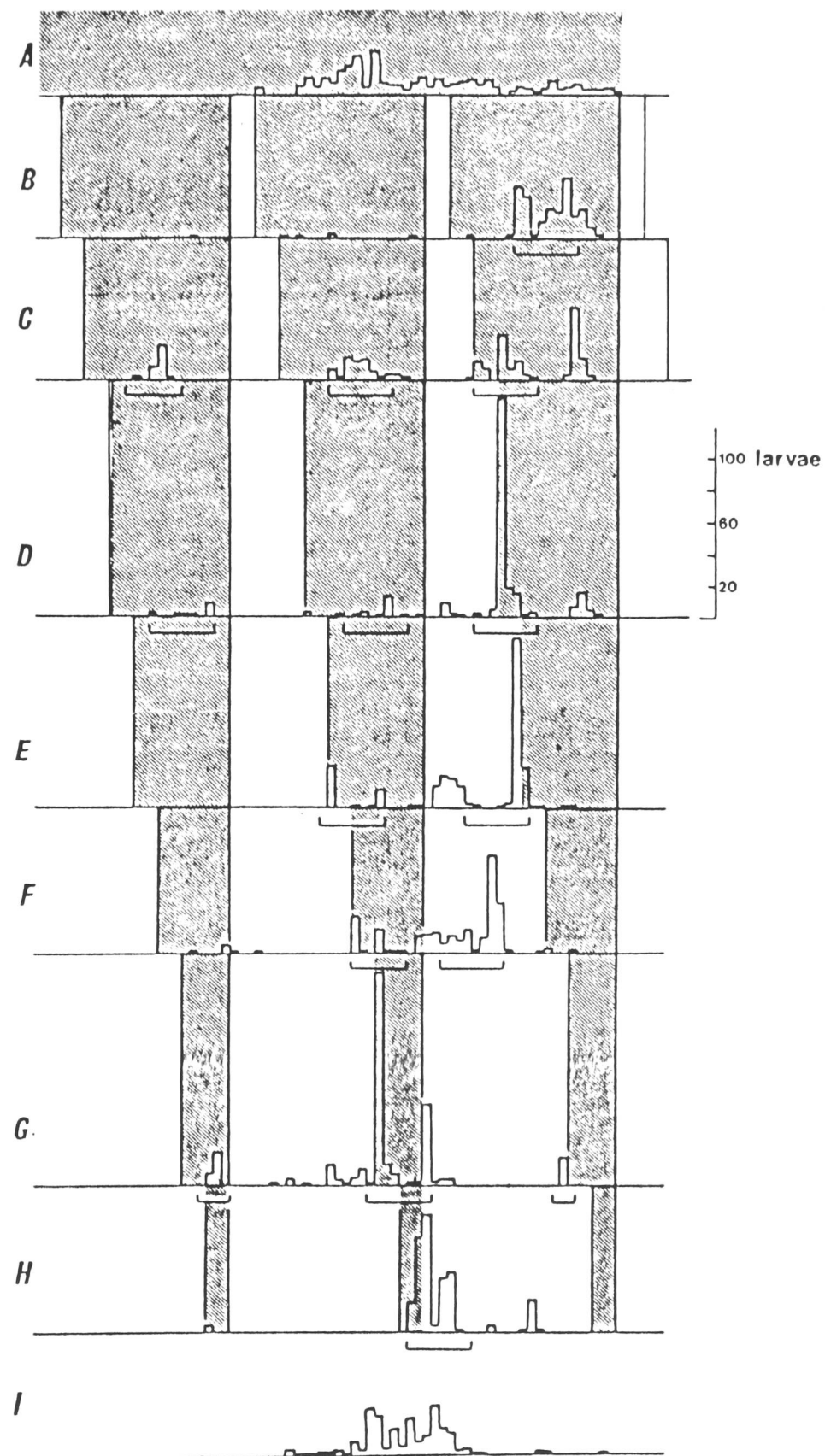


Figure 2.2. Larval wandering behaviour in nine cultures (A-I) exposed to light cycles of DD;LD 3:21, 6:18, 9:15, 12:12, 15:9, 18:6 and 21:3; and LL respectively (with the lights-on transition occurring at the same local time). Horizontal brackets mark the most dense 8h of activity in each peak.

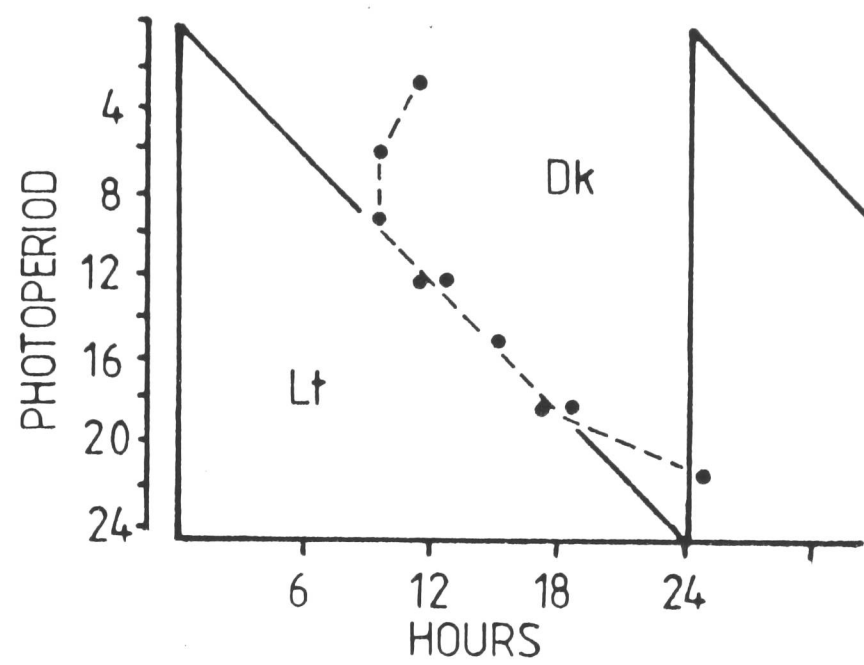


Figure 2.3. Phase relationship of larval wandering to complete photoperiods. Each point is the mean position of the larval wandering gates in relation to the photoperiods as taken from figure 3.2. Lt, light period; Dk, dark period.

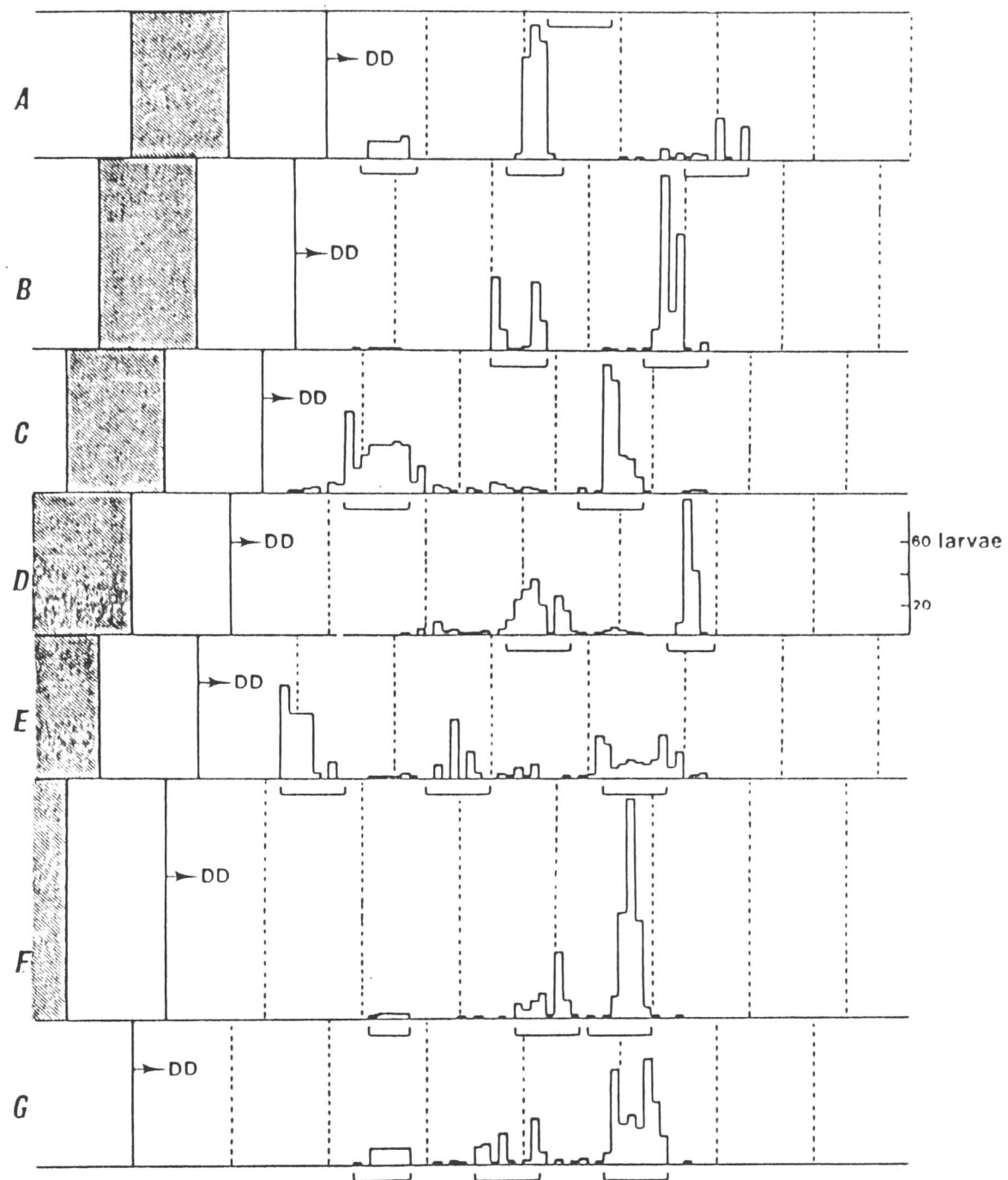


Figure 2.4. Larval wandering behaviour in seven cultures (A-G) released from three cycles of LD 12:12 into continuous darkness (DD) at different local times. Horizontal brackets mark the most dense 8h of activity in each peak. Vertical dotted lines mark 12h intervals from the onset of DD.

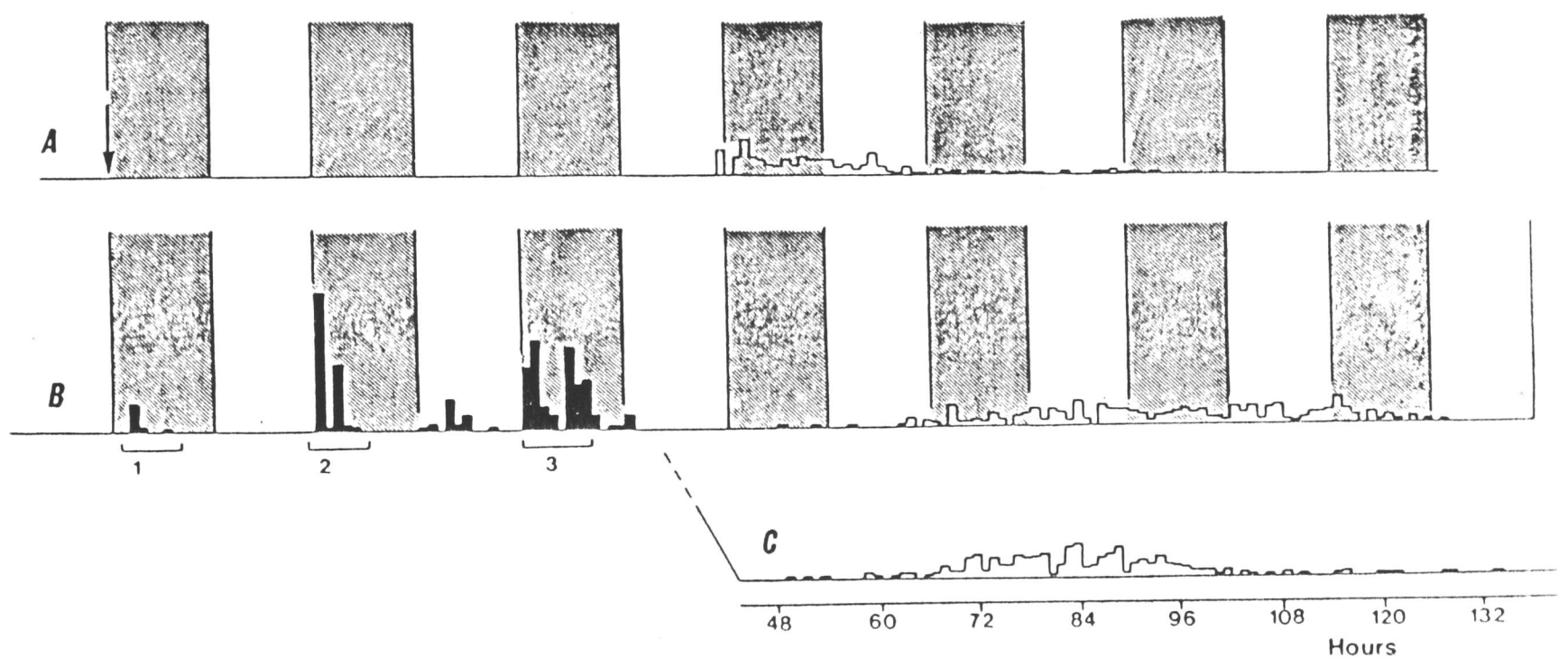


Figure 2.5 (A) Distribution of puparium formation in a mixed-age culture of *S. argyrostoma* raised in LD 12:12, 20°C whose fully fed larvae were manually extracted from their food (at arrow). (B) Larval wandering (solid histograms) and puparium formation (open histograms) in a culture allowed to wander naturally. (C) Distribution of puparium formation in culture B plotted in hours from larval wandering. Horizontal brackets in B mark the most dense 8h of activity in each peak.

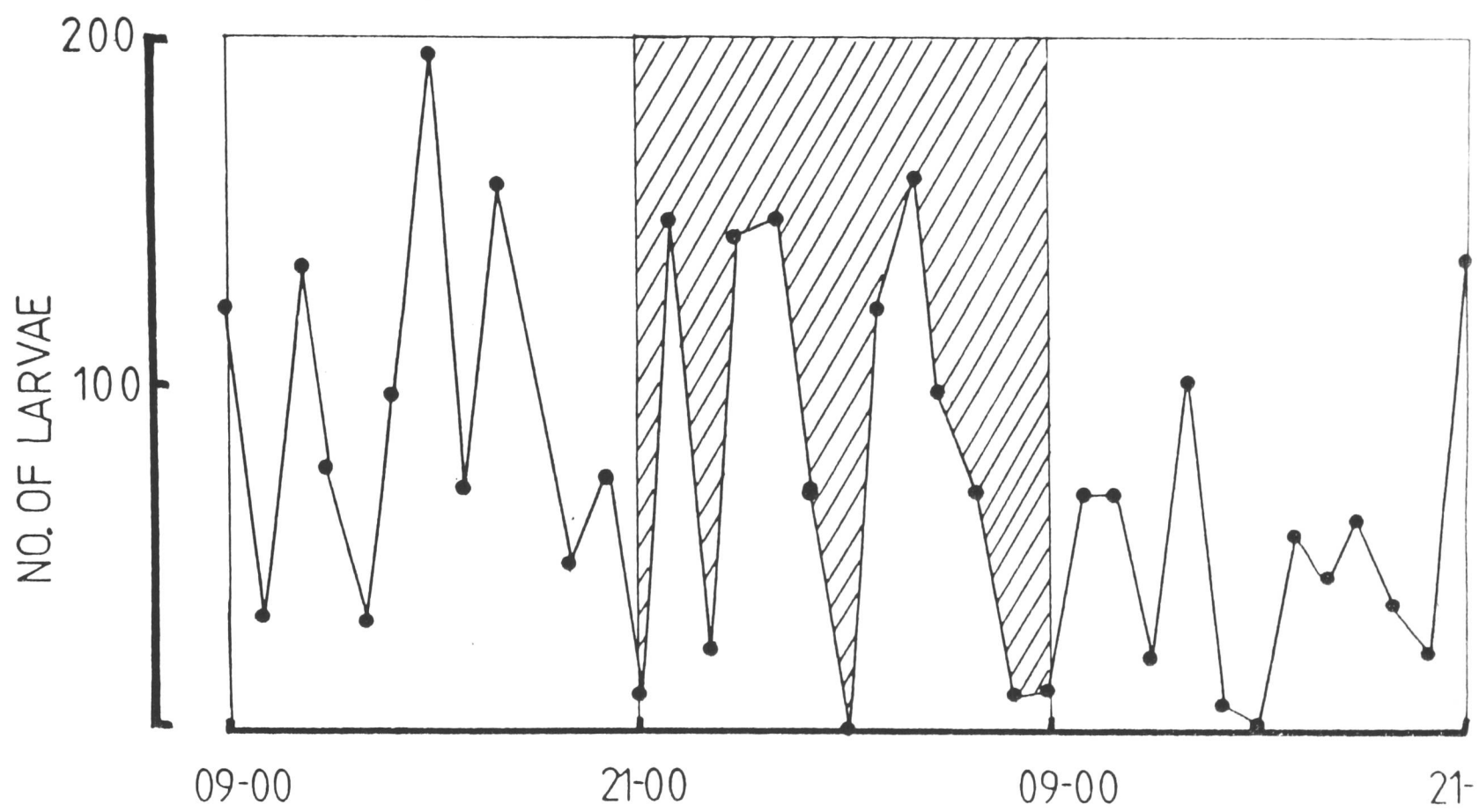


Figure 2.6. Larviposition by females of *S. argyrostoma* in LD 12:12 at 25°C showing deposition of larvae at all times of the day or night.

## Discussion

These results show that the onset of wandering behaviour in fully fed larvae of *Sarcophaga argyrostoma* occurs largely at night or shortly after lights-off. In a population therefore, exodus is rhythmic with a series of nocturnal peaks of activity similar to those described for the blowflies *Lucilia cuprina* and *Chrysomya bezziana* (Smith *et al.*1981; Spradberry *et al.*1983) and for the flesh fly *Sarcophaga bullata* (Roberts,1984). In *S. argyrostoma* this rhythm is partly governed by the circadian system so that it free-runs in continuous darkness with an endogenous period close to 21h. Since each larva leaves its food only once, a mixed age population is required to demonstrate such a rhythm; the rhythm is therefore 'gated' as in pupal eclosion (Pittendrigh,1954; Saunders,1976). In marked contrast, pupariation and larviposition are evidently not gated and can occur at any time of the day or night.

When compared with pupal eclosion in *Sarcophaga* (Saunders,1976), it is clear that the circadian regulation of larval exodus is rather weak, with some of its peaks being poorly defined, and an ability to free-run in darkness being restricted to those cultures recently exposed to several light- dark cycles. When cultures of newly deposited *S. argyrostoma* larvae were exposed to a simple step-down from LL to DD, the eclosion rhythm manifested itself 16 to 21 days later with a mean free-running period of 23.8h (Saunders,1979). The present investigation has shown that the exposure of newly deposited larvae to a similar step-down is not sufficient to induce wandering periodicity. This may reflect either a rapid damping of the oscillatory system over the feeding

period or a developing asynchrony between the rhythms of individual larvae. Bearing in mind the ability of populations of *Sarcophaga* to maintain the synchrony of their eclosion rhythms over a much longer period of time than this, it may be more likely that the wandering rhythm is damped down during the feeding period.

Working with *S. bullata*, Roberts (1984) demonstrated that the nocturnal peak of larval wandering coincided with a pulse of ecdysteroids in the haemolymph, itself immediately preceded by a heightened synthesis and release of ecdysone from the ring gland (as measured *in vitro*). Chapter 3 will present data showing that the onset of larval wandering in *S. argyrostoma* is also preceded by a peak of ecdysteroid activity in the haemolymph. These increases in titre were presumably the result of even earlier releases of prothoracicotropic hormone (PTTH) from the brain. It is this release of PTTH that is thought to be the primary gated event. In the tobacco hornworm *Manduca sexta*, it has been shown that the pulsatile release of PTTH prior to larval exodus is a gated event, but that before pupariation is not (Truman and Riddiford, 1974; Gilbert *et al.*, 1980; Dominick and Truman, 1984). Although the neuroendocrine events governing gated mechanisms are largely unknown, it is possible that the cerebral circadian clock issues a daily signal which leads to PTTH release only when other factors permit. Since a high titre of juvenile hormone (JH) is known to inhibit PTTH release in final instar *M. sexta* larvae (Nijhout and Williams, 1975), a drop in the titre of this hormone may be the permissive factor involved. Walker and Denlinger (1980) have demonstrated that JH levels in diapausing *Sarcophaga crassipalpis* pupae rise and fall on a daily basis. It will be shown in chapter 4 of this thesis that PTTH levels in the brain of diapausing *S. argyrostoma* pupae are high and that it is presumably not being released. Evidence suggests therefore that JH may inhibit the

release of PTTH in *Sarcophaga* spp. in diapause. Consequently, it may indeed be a factor involved in the gated release of PTTH prior to the onset of wandering behaviour.

It is interesting to compare developmental events in *S. argyrostoma* which are known to be clock controlled (larval exodus and pupal eclosion) with those that are not (pupariation and larviposition), and to consider the possible selective advantages for such control. Clearly there can be little advantage gained by forming the puparium at a certain phase of the light-dark cycle since the larva is already underground at this stage. Chapter 1 has shown that puparium formation can be delayed by crowding the post-feeding larvae. Ohtaki (1969) reported that *S. peregrina* larvae will delay pupariation if maintained in wet conditions. Presumably therefore, pupariation will be delayed until suitable conditions have been found. The wide spread of pupariation times reported in this chapter, from populations of larvae maintained under the optimum conditions (dry and singly in tubes with sawdust), probably results from differences between individual larvae. In addition, the larvae in this portion of the investigation were raised under an LD 12:12 diapause-inducing photoperiod which has already been shown to delay pupariation when compared to larvae raised under non-diapause inducing photoperiods (chapter 1). There is no apparent advantage to larviposition being a gated event in this species which would normally deposit larvae on carrion found in the wild. This study noted that larviposition occurred at all times of the day or night. However, Saunders (1986) cites unpublished observations that adult locomotor activity is strongest during the day, and by analogy with other species, *Phormia terraenovae* and *Lucilia cuprina* (Aschoff and Saint Paul, 1982; Smith, 1983), may have a circadian component. If this is the case, it is puzzling that larviposition which would require a degree of locomotor activity, does not



display any association with a particular phase of the photoperiod. This result may be an artifact of maintaining adult flies in a caged environment in close proximity to the meat supplied for larviposition.

Pupal eclosion and larval wandering, on the other hand, are both gated, but differ in both phase and circadian period, the former occurring close to dawn and showing a free-running period close to 24h (Saunders, 1976), and the latter occurring close to dusk with a period of about 21h. The selective advantage of flies emerging at dawn might be that wing expansion is more effectively accomplished at high humidity (Pittendrigh, 1958), or merely that a mass dawn emergence synchronizes a population of diurnally active flies for the day ahead. For eclosion, a circadian period of close to 24h enables the developing adult flies, lying underground for several weeks, to emerge at the most appropriate time, measured in multiples of 24h since the last seen dawn.

The advantages of dusk or nocturnal wandering are more problematical. Roberts (1984) argued that such behaviour enables the larvae to avoid diurnal predators, those larvae waiting for the next gate within the carcass enjoying a safe refuge. On the other hand, nocturnal wandering may expose the larvae to a different set of predators, and staying within the carcass during the day merely provide a convenient focus for carrion-feeding birds. The selective advantage of nocturnal wandering could lie in the avoidance of desiccation.

The clock governing pupal eclosion is phase-set by light during embryonic and early larval development, and runs with an endogenous period close to 24h (Saunders, 1976). The clock governing larval wandering is probably also entrained during the same developmental stages, but then shows a much shorter period (about 21h). The two systems therefore possess different properties. This raises the possibility that they are regulated by two separate

circadian oscillators, rather like those described for pupal eclosion and adult locomotor activity in *Drosophila pseudoobscura* (Engelmann and Mack, 1978).

The role of the circadian system in the behaviour and development of *Sarcophaga argyrostoma* has been reviewed by Saunders (1986). He proposes that these events may be controlled by at least three and possibly six separate circadian clocks. Pupal eclosion and larval wandering which display free-running periods of about 24 and 21h respectively have already been discussed here. The induction of diapause is determined by the exposure of embryos and larvae to a number of nights longer than 9.5h (the critical night length) which are accumulated by a photoperiodic counter (Saunders, 1981). Both the clock which measures night length and the counter are thought to be circadian in nature, and to depend upon the entrainment of the circadian system to the photoperiod. Saunders (1986) suggests that the photoperiodic pacemaker determining the induction of diapause operates with a period of 25h and may be rapidly damped out in the absence of photoperiodic cues. The duration of larval wandering may be under some photoperiodic control since exposure to diapause inducing photoperiods causes the wandering period to become protracted. The critical photoperiod for wandering is probably close to that for pupal diapause induction (Saunders, 1976). The length of the wandering period can be affected by photoperiod even in non-diapause inducing conditions; exposure to LL at 25°C can also delay pupariation. The duration of this period and diapause induction can therefore be separated experimentally suggesting that they may be under the control of separate circadian systems. Lukat (1978) noted that the regulation of daily growth layers on thoracic apodemes persists in cockroaches rendered behaviourally arrhythmic by the bilateral isolation of the optic lobes. This is presumably under the control of a separate circadian oscillator. Finally adult locomotion may be under the control

of the circadian system. Events which have been shown not to be under the control of the circadian system include larviposition, larval moults (see chapter 1) and pupariation. Non-photoperiodically regulated events include larval development up to the wandering period and ovarian and embryonic development.

## CHAPTER 3

Haemolymph ecdysteroid titres in diapause and non-diapause destined *Sarcophaga argyrostoma*

**Haemolymph ecdysteroid titres in diapause and non-diapause****destined *Sarcophaga argyrostoma*****Introduction**

This chapter will begin the investigation into the endocrinology of diapause in *Sarcophaga argyrostoma*. It has long been proposed that diapause in many insect species may result from the absence, or presence, of a factor or factors normally required for, or antagonistic to, continued development. Wigglesworth (1934,1936) first suggested that diapause is directly caused by the failure to secrete specific hormones. This concept that diapause is mediated by the neuroendocrine system was developed further by Williams (1946,1947,1952) working on the pupal diapause of the cecropia silkworm *Hyalophora cecropia*, by Fukuda (1951,1952) and Hasegawa (1951,1952) working on the egg diapause of the commercial silkworm *Bombyx mori*, and by de Wilde's (1953,1954) experiments on the adult diapause of the Colorado beetle *Leptinotarsa decemlineata*.

Diapause resulting from the failure of the neuroendocrine system to continue development was demonstrated by the pioneering experiments of Williams (1946,1947,1952) who showed that the brain failed to supply a non-species specific factor, now known to be the prothoraciotropic hormone (PTTH), which activated the prothoracic gland to produce a moulting hormone. Similar forms of endocrine control have been reported in the larval diapauses

of the European corn-borer *Ostrinia nubilalis* (Cloutier *et al.*,1962), the blowfly *Lucilia caesar* (Fraser,1960) and the wheat stem sawfly *Cephus cinctus* (Church,1955), and in the pupal diapauses of the Bertha armyworm *Mamestra configurata* (Bodnaryk,1977), the lime hawk moth *Mimas tiliae* (Highnam,1958) and the fleshfly *S. argyrostoma* (Fraenkel and Hsiao,1968b).

In a number of Lepidopteran species, this simple hormone-failure theory does not adequately explain larval diapause. In the southwestern corn borer *Diatraea grandiosella*, diapause is not caused by the lack of the moulting hormone but rather by high titres of the juvenile hormones JHI, II, and III in the haemolymph during diapause (Yin and Chippendale,1973; Bergot *et al.*, 1976). High JH titres in the final larval instar of the rice stem borer *Chilo suppressalis* (Yagi and Fukaya,1974) and the codling moth *Laspeyresia pomonella* (Sieber and Benz,1977) induce larval diapause; in the former species they are actually required for its maintenance. Takeda (1978) demonstrated that during the early stages of prepupal diapause in the slugmoth *Monema flavescens*, the corpus allatum was actively releasing JH whilst the neurosecretory cells in the brain appeared to be inactive. In an attempt to explain the role of JH in the regulation of larval diapause, Nijhout and Williams (1974) demonstrated that high levels of JH in the final larval instar of the tobacco hornworm *Manduca sexta* inhibited the release of PTTH. Once JH was absent, PTTH release resumed and pupation occurred. They postulated therefore that JH was causally involved in the the larval diapauses of at least some Lepidoptera. Walker and Denlinger (1980) showed that JH may be involved in the pupal diapause of *S. crassipalpis*. These workers noted cyclic pulses of JH activity in the haemolymph of diapause destined prepupae, which continued into diapause (unpublished results, cited in Denlinger,1985). These pulses, which occurred with a period of approximately 24h, appeared to be

negatively correlated with periods of high oxygen consumption by the diapausing pupae, indicating a role for JH in the regulation of metabolism during diapause.

In the silkworm *Bombyx mori*, embryonic diapause is determined by the presence of a 'diapause hormone' (DH) produced by the neurosecretory cells of the suboesophageal ganglia of moths reared from eggs and young larvae exposed to long days (Hasegawa, 1957). The hormone has been shown to exist in two forms (molecular weights 3300 and 2000), both based on a 14 amino acid peptide associated with two types of amino sugars (Isobe *et al.*, 1975). Kobayashi and Ishitaya (1964) demonstrated that the production of DH is controlled by the brain and that the target organ is the ovaries. It appears to be incorporated into the ovarian eggs and imposes diapause on the embryos after oviposition. A similar developmental mechanism has been proposed by Kind (1965) for the embryonic diapause in the Tussock moth *Orgyia antiqua*. Andrewartha *et al.* (1974) provided evidence that the pupal diapause in *Phalaenoides glycinae* may also be initiated by a hormone from the suboesophageal ganglion. This hormone was shown to be capable of inducing diapause in *B. mori* eggs when ganglia from moths kept under diapause inducing conditions were transferred to the silkworm. However, some species that totally lack a diapause stage, such as the American cockroach *Periplaneta americana*, also exhibit DH activity (Takeda, 1977). It may be that DH in *P. glycinae* has no regulatory role in pupal diapause.

Giebultowicz and Saunders (1983) reported that they were unable to detect the presence of a diapause inducing hormone in *Sarcophaga argyrostoma*. Indeed, no such hormone has been found in any short-day insects, *i.e.* those insects in which diapause is induced by exposure to short days during the

sensitive period. For all species with a post-embryonic diapause, with the possible exception of *P. glycinae*, Wigglesworth's (1934,36) theory of hormonal failure remains appropriate, despite the fact that the regulatory mechanisms controlling diapause in these stages appears diverse. In all cases, diapause results from the inactivation of neurosecretory cells within the brain.

Before a consideration of the hormonal mechanisms of pupal diapause induction in *Sarcophaga* species can be given, a brief account of the endocrinology involved in normal development, particularly PTTH and ecdysone, is necessary.

The prothoracicotropic hormone (PTTH) is the primary effector in the neural transduction of external (photoperiod) and internal (proprioception) cues to the endocrine elements controlling insect moulting (Gilbert *et al.*,1981.) The hormone, or more accurately hormones, since two forms have been identified (Bollenbacher *et al.*,1984), is produced in *Manduca* by a pair of neurosecretory cells located in the protocerebrum (Agui *et al.*,1979). It is transported axonally to the corpus allatum which has been identified as the neurohaemal organ for this hormone (Agui *et al.*,1980) and released to exert its' trophic effect on the prothoracic glands. This is reviewed in greater detail in chapter 4 where the mechanisms of PTTH action are considered.

The control of the synthesis and the release of PTTH from the neurosecretory cells is not fully understood. Several stimuli can elicit the synthesis and/or the release of PTTH, including nutritional state, crowding, wounding, and especially photoperiod and temperature (Doane,1972). This worker suggested that whilst the neural pathways of transduction are unknown, control may be exerted indirectly via synaptic inhibition or stimulation by other neurones, or directly on the neurosecretory cells themselves. In some



cases, the release of PTTH does not appear to be under the control of the brain's photoperiodic or circadian clock mechanisms (for example, pupariation, see chapter 2), whereas in at least one instance, release is clock-controlled (for example, larval wandering behaviour, see chapter 2). In the pupae of the oak silkmoth *Antheraea pernyi*, the photoperiodic mechanisms effecting PTTH action lie in a lateral area of the brain and control release rather than synthesis (Williams, 1969). Even when normal neural activity is interrupted by the injection of tetrodotoxin, which acts by blocking sodium activation of nerve membranes thereby inhibiting interneural transmission, these pupae respond normally to varying photoperiodic regimes. It is possible therefore that the photoperiodic machinery regulating PTTH release may reside within the PTTH neurosecretory cells themselves.

It has been suggested that certain humoral factors may elicit or inhibit PTTH synthesis and release. For example, the corpora allata of the rice stem borer *Chilo suppressalis* are active during larval diapause, and JH was shown to inhibit the release of PTTH (Fukaya and Mitsuhashi, 1961; Yagi and Fukaya, 1974). Similar mechanisms have been shown to exist in other species; Takeda (1978) and Yin and Chippendale (1973) have extended this concept to prepupal diapause in *Monema flavescens* and larval diapause in *Diatraea grandiosella* respectively. The mechanisms of PTTH release termination and subsequent preparation for the next period of release are thought to be complex (Gilbert, 1980). Observations of hormonal effects on cerebral neurosecretion suggest that release and/or synthesis are probably regulated at various levels by both JH and the moulting hormone, and even perhaps by other factors (Adams, 1975), possibly from within the brain.

The trophic effect of PTTH is exerted on the prothoracic glands resulting in

the synthesis of the moulting hormone. The prothoracic glands appear to be the only physiological source of this hormone during larval/larval and larval/pupal development (King *et al.*,1974) even though other tissues can apparently synthesize it (Galbraith *et al.*,1973; Nakanishi *et al.*,1972). Ecdysone is the ecysteroid synthesized by the prothoracic glands of most insect species (Bollenbacher *et al.*,1976; Gilbert *et al.*,1977) and now appears to be the primary ecdysteroid produced by larval glands (Chino *et al.*,1974; King *et al.*,1974). However, Redfern (1984) did note that under certain dietary conditions, the prothoracic glands of *Drosophila melanogaster* could be induced to produce 20-deoxymakisterone A instead of ecdysone.

Ecdysteroids are polyhydroxylated ketosteroids (See figure 3.1), all having the characteristic tetracyclic nucleus. With the exception of the 28- carbon makisterone A and its derivatives, and the 30-carbon lobosterol, all zooecdysteroids are 27-carbon steroids (Horn and Bergamasco,1985). They vary only in the number and/or stereochemistry of the hydroxyl groups present (Morgan and Pool,1977). In contrast to vertebrate animals and plants, insects cannot synthesize sterols *de novo* from small molecules such as acetate or mevalonate (Rees,1985). Consequently, for normal growth, development and reproduction, insects require a dietary intake of sterols. Whilst carnivorous insects can obtain cholesterol directly from their food, thereby fulfilling their requirements, the natural dietary sterols of phytophagous species are mostly ones bearing an alkyl group at C-24, for example, sitosterol, stigmasterol and campesterol. These sterols require dealkylation to cholesterol prior to the synthesis of ecdysone. Most species investigated are able to effect these reactions, but three, the large milkweed bug *Oncopeltus fasciatus*, the Khapra beetle *Trogoderma granarium* and the honey bee *Apis mellifera* (Svoboda *et al.* 1977,1980,1981), are apparently

incapable of these processes. Presumably these species have no major requirement for C-27 sterols. In the case of the C-28 ecdysteroid makisterone A, isolated from the eggs of *Oncopeltus* (Kaplanis *et al.*, 1975), the precursor would be expected to be a C-28 sterol such as campesterol. Redfern's (1984) observations on 2-deoxymakisterone A production by *Drosophila* presumably indicates that in some species, both C-27 and C-28 sterol precursors can be used depending upon availability.

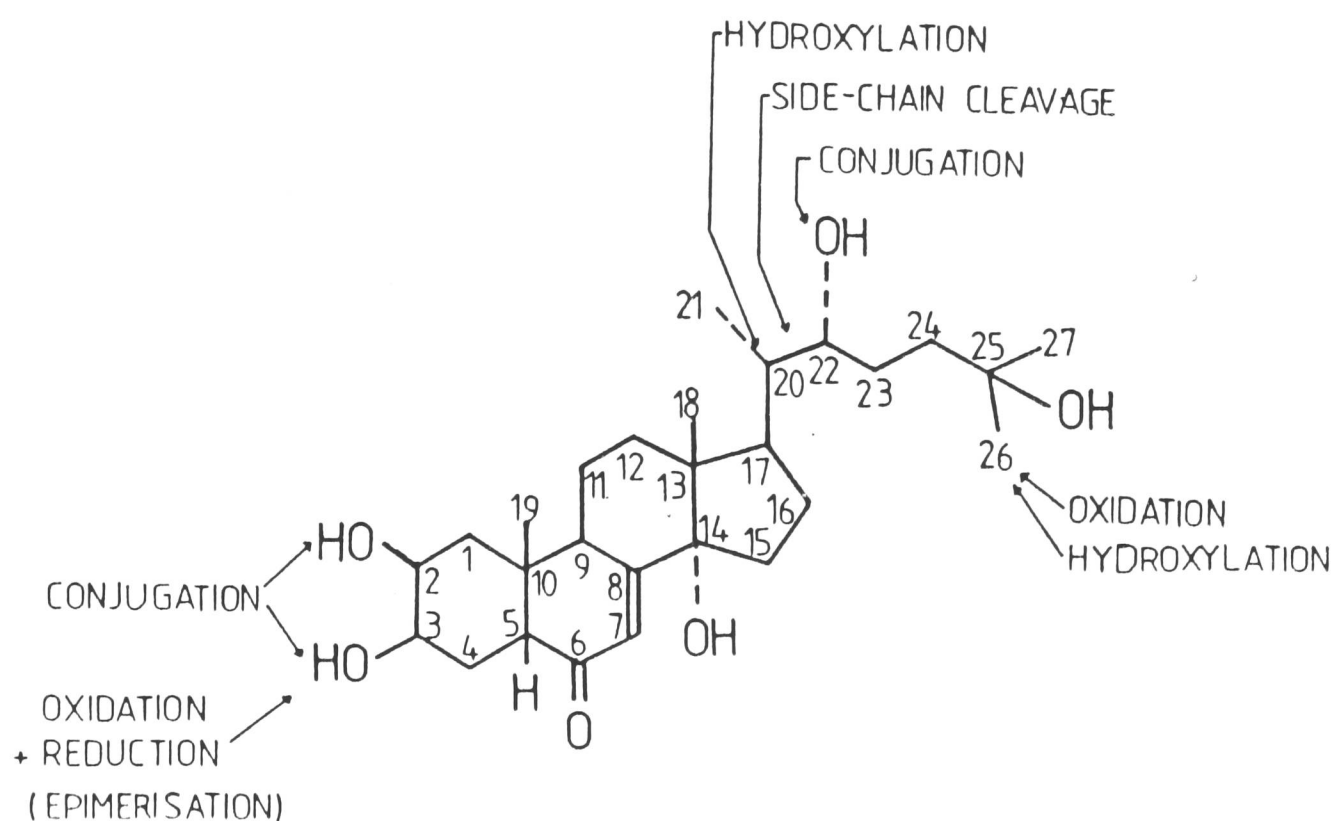


Figure 3.1: The chemical structure of ecdysone showing the various catabolic reaction positions. The numbers refer to the position of the carbon atoms involved in the tetracyclic nucleus and side chain structure.

Investigations into the biosynthetic pathways of ecdysteroids have primarily focussed on the synthesis of ecdysone and 20-hydroxyecdysone during larval/pupal development (Gilbert *et al.*,1980). An *in vivo* approach has generally been used in which possible sterol precursors are injected into an insect and the metabolites then analysed. These studies indicated that several biosynthetic pathways exist, even within a particular species, at different developmental stages. Svoboda *et al.* (1975) demonstrated that a primary route for the early steps in ecdysone synthesis involved the saturation of the  $\Delta^5$  bond of cholesterol, establishment of a  $\Delta^7$  bond, the addition of a 6-keto group and *cis* fusion of the A/B rings of the tetracyclic nucleus. Later steps involve hydroxylations at C-14, C-2, C25 and ultimately C-22 to form ecdysone (Gilbert *et al.*,1977; Svoboda *et al.*,1975).

An *in vitro* approach to ecdysone biosynthesis has so far corroborated the conclusions from the *in vivo* studies (Bollenbacher *et al.*,1977b). Since this technique has allowed the analysis of individual steps in the pathways, additional information into the early steps has been obtained. For example, a monohydroxylated 5 $\alpha$ -analogue of ecdysone has been identified in silkworm larval prothoracic glands *in vitro* which may function as an early biosynthetic intermediate (Sakurai *et al.*,1977). In *Manduca* larvae, the precursor for ecdysone synthesis by the prothoracic gland may be a more highly oxygenated steroid than either cholesterol or the 5 $\alpha$ -analogue (Bollenbacher *et al.*,1977b). These two schemes are not compatible with a common biosynthetic mechanism which suggests that ecdysone synthesis may differ even in insects of the same order.

The mechanisms regulating ecdysone synthesis and release from the prothoracic glands are discussed in chapter 4 where an investigation into

PTTH action and the involvement of a cyclic nucleotide secondary messenger system is presented.

Ecdysone, when released from the prothoracic glands, can be hydroxylated to 20-hydroxyecdysone in specific peripheral tissues (King and Siddall, 1969; King, 1969, 1972a, 1972b). This is the only step in the biosynthesis of 20-hydroxyecdysone from cholesterol in which partial characterisation of the enzyme systems involved has been achieved (Smith, 1985). There are several reasons why this reaction has been so widely studied. The availability of high specific activity radiolabeled ecdysone has allowed trace detection and quantification of the reaction products (King and Siddall, 1968; Haffner *et al.*, 1972); it is a single hydroxylation reaction so information obtained here can be used to elucidate other more complex hydroxylation reactions involved in the conversion of cholesterol; and most importantly, the potential biological significance of this reaction which produces the active moulting hormone has helped to focus attention upon it. The conversion is effected by ecdysone-20-monooxygenase, a steroid mixed function oxidase catalysed by a cytochrome P-450 system associated with NADH and NADPH generating systems (Smith, 1985). Studies by King (1969, 1972a, 1972b) on several insect orders demonstrated that the major tissue sources of monooxygenase activity were the fat body, the Malpighian tubules, the midgut, integument and possibly the gonads. The subcellular location of enzyme activity appears to be both mitochondrial and microsomal, dependent on the species (Gilbert *et al.*, 1980). The rate of conversion of ecdysone to 20-hydroxyecdysone has been shown to vary throughout development in some species, apparently following the titre of the moulting hormone (Smith, 1985).

The active ecdysteroid titre can therefore be regulated by a combination of

several biosynthetic mechanisms in response to PTTH stimulation of the prothoracic glands. The rate of conversion of ecdysone to its active form can also be regulated thereby modulating the effects of PTTH. A further regulatory mechanism exists which has not yet been discussed, that of ecdysteroid catabolism. Koolman and Karlson (1985) review this area of ecdysteroid biochemistry and highlight three basic forms of metabolism involved in the deactivation of 20-hydroxyecdysone. These are further hydroxylation often followed by oxidation, oxidation and/or epimerisation (isomerisation), and conjugation.

Although the hydroxylation of ecdysone at C-20 yields a product with greatly enhanced activity, further hydroxylation at C-26 yields a product with reduced hormonal capacity (Bergamasco and Horn, 1980). This hydroxylation can therefore be regarded as the first stage of deactivation. When ecdysone and 20-hydroxyecdysone are so hydroxylated, the inactive forms 26-hydroxyecdysone and 20,26-dihydroxyecdysone are produced. Further inactivation may be effected by the subsequent oxidation of the C-26 hydroxyl groups to the corresponding carboxylic acids (Koolman, 1982), ecdysonic acid and 20-hydroxyecdysonic acid respectively. Lafont *et al.* (1980) suggest that similar oxidation products may be expected directly from 20-hydroxyecdysone and possibly from the epimers 3-epiecdysone and 3-epi-20-hydroxyecdysone. These epimers are themselves the products of an oxidation/reduction reaction which serves to deactivate the ecdysteroids without any further hydroxylation.

An analysis of haemolymph ecdysteroids by high performance liquid chromatography reveals a group of highly polar products which contain the carboxylic acids mentioned above and a larger mass of unspecified ecdysteroid material. After incubation of this fraction with hydrolysing enzymes, a mixture

of free ecdysteroids is recovered (Koolman,1985), indicating that many types of ecdysteroids can be chemically bound (conjugated) to other chemical groups, possibly to effect deactivation. Three groups of conjugates have been discovered so far. The presence of sulphate esters of ecdysteroids has been established by the incorporation of [<sup>35</sup>S] *in vivo* after the administration of radiolabelled sulphate (Koolman *et al.*,1973). Phosphate esters, such as ecdysone-22-phosphate and 2-deoxyecdysone-22-phosphate have been identified in some species (Issac *et al.*,1982). More complex esters such as the 22-ester of 2-deoxyecdysone with adenosine monophosphoric acid have been discovered in the eggs of *Locusta migratoria* (Tsoupras *et al.*,1982). The third group was discovered by Heinrich and Hoffmeister (1970) who reported the isolation and identification of an  $\alpha$ -glucoside of 20-hydroxyecdysone in the highly polar fraction. However, the presence of other glucosides and glucuronides have not yet been convincingly demonstrated by other authors.

The reverse of these conjugation processes, the hydrolysis of conjugates *in vivo*, has often been postulated and indeed, indirect evidence has been obtained from a few insect species (Koolman,1985) in which ecdysteroids can be released from a certain fraction of the conjugates. This would infer that there are two types of ecdysteroid conjugates, inactivation products and storage forms.

The metabolic end products outlined here are normally excreted either as free ecdysteroids or as conjugates; both have been found in the faeces of various insect species. Gee *et al.* (1977) observed that ecdysone and 20-hydroxyecdysone were both excreted by the Malphigian tubules of the tsetse fly *Glossina morsitans*. During developmental stages which do not possess an active excretory system (for example, eggs, embryos and pupae), active

ecdysteroids have to be removed by means other than excretion, usually by either deactivation or by compartmentalisation. Koolman (1978) noted efficient inactivation by ecdysone oxidase, ecdysone-3-epimerase and by conjugating enzymes in the pupae of *Calliphora vicina*. In *Manduca* however, ecdysone and its metabolites were removed from the circulatory system (Kaplanis *et al.*, 1980a) with high ecdysteroid concentrations being detected in the pupal meconium. This appears to function as a depository for these waste products.

Ecdysteroids have been measured in numerous species of insects during various stages of postembryonic development. Smith (1985) gives a comprehensive review of the major species in which titres have been determined. Despite differences between species, there are several generalizations and patterns that have emerged from these studies. Ecdysteroids are always present during these developmental stages, albeit in widely varying concentrations ranging from low picogram per microlitre of haemolymph to high nanogram levels. Within any one species, the level can vary from the basal levels by factors of 20–30, in the case of *Drosophila melanogaster*, to  $10^2$  to  $10^3$  in *Manduca sexta* (Richards, 1981). These dramatic changes do not appear to occur randomly. At least one major peak in the ecdysteroid titre has been found during every insect larval or pupal stage examined. Bollenbacher *et al.* (1981) demonstrated that a large peak of ecdysteroids occurred during both the fourth and fifth instars of *Manduca*, immediately prior to the onset of the larval/larval and larval/pupal moults respectively. Dean *et al.* (1980), using data from a range of sources, demonstrated that peaks occurred prior to larval moults in eight different Lepidopteran species. During the pupal stages of seven species, these workers also demonstrated that a large pulse of activity occurred shortly after pupation. The size and duration of this peak varied but it was present in all



species examined.

The major peak in ecdysteroid titre associated with the moult usually occurs towards the end of the instar. It is characterised by a sharp rise from basal levels before apolysis, maximal levels either slightly before or after apolysis and an equally rapid drop by the time of full ecdysis (Smith,1985). Despite differences between species, it appears that the peaks preceding larval/pupal and pupal/adult moults are of slightly greater magnitude than those preceding larval/larval moults. There are of course exceptions to this general pattern. In *Calliphora vicina*, the major peak associated with the onset of pupariation does not drop back to basal levels until pharate adult development is almost complete (Koolman,1980). During pupal/adult development in many species, the major peak occurs in the first half or the middle of the stage (Dean *et al.*,1980). This peak is characterised by a slow rise from basal levels with the onset of pupal/adult apolysis, followed by a period of sustained release and then a slow return to basal levels before pupal/adult ecdysis. However, Wentworth *et al.* (1981) reported that two major peaks occur in the pupal stage of *Sarcophaga bullata*, and in the waxmoth *Galleria mellonella*, the major peak early in pupal development is followed by another immediately prior to pupal/adult development (Bollenbacher *et al.*,1978). Whilst it may not seem surprising that differences exist between species, it should be borne in mind that Richards (1981) in his review of eight titre studies of *Drosophila melanogaster* by various authors, noted that eleven distinct hormone peaks were suggested, although no one study demonstrated more than five. Another cautionary note regarding most of the studies reported to date is that there is no information regarding the composition of the ecdysteroid peaks. Most studies have used either bioassay methods which only measure the total active component within the system, *i.e.* the level of

ecdysteroids which are capable of effecting a response, or they have used radioimmunoassay techniques with antisera not capable of distinguishing between the different ecdysteroids present. The results in these latter studies are generally expressed either as ecdysone equivalents or 20-hydroxyecdysone equivalents, depending upon the ecdysteroid used to calibrate the assay system.

The postembryonic whole body ecdysteroid titres in *Sarcophaga bullata* have been examined in some detail by Wentworth *et al.* (1981). These workers noted that peaks of ecdysteroid activity occurred prior to each larval/larval moult, and a larger peak prior to pupariation. Twenty-four hours following pupariation, an increase many times larger than the moulting peaks occurred prior to pupal apolysis. The level then fell before the onset of two even larger peaks during pharate adult development. This rise and fall of the titre after pupariation was also found in *S. crassipalpis* by Walker and Denlinger (1980) who used an ecdysteroid bioassay rather than a radioimmunoassay. Similar data was provided by Briers *et al.* for *Calliphora vicina*, *Phormia terrae-novae* and *Lucilia caesar*. The profile of ecdysteroid titres at this stage of development appears therefore to be relatively uniform in a number of species of Diptera. A possible ecdysteroid mediated event which was not determined by Wentworth *et al.* (1981) or by Walker and Denlinger (1980) is that of larval wandering, as discussed in chapter 2. Bollenbacher and Gilbert (1981) noted a small rise from the basal ecdysteroid titre in *Manduca* prior to the onset of this wandering behaviour. In addition to the involvement of this rise in the induction of wandering behaviour, Riddiford (1976) suggested that in *Manduca sexta*, it may change the commitment of the tissues from larval to pupal syntheses in preparation for the next major peak of ecdysteroids responsible for the induction of puparium formation. Bollenbacher and Gilbert (1981) have

associated increases in ecdysteroid titres with specific pulses of PTTH release. The pulse prior to wandering actually appears to be three very short bursts of PTTH release. They further demonstrated the association of PTTH release with the ecdysteroid peaks responsible for larval/larval and larval/pupal moults.

The traditional view of the endocrinology of diapause involves a failure of the endocrine system to continue development beyond a particular stage. This view appears to be largely applicable to the pupal diapause of *Sarcophaga* species which enter diapause prior to pupal/adult apolysis (Fraenkel and Hsiao, 1968a). Walker and Denlinger (1980), using a *Musca* bioassay, demonstrated that *Sarcophaga crassipalpis* whole body ecdysteroid titres fell as the pupae entered diapause, as compared to titres from those pupae undergoing continuous development. In non-diapause destined pupae, the first increase and reduction of ecdysteroid levels is followed within 24–48h by a second surge which initiates adult development. In diapause destined pupae however, this second surge does not occur and the titre drops to below the level of detection of the bioassay.

Juvenile hormone may also play an important functional role prior to the onset of diapause (Walker and Denlinger, 1980). At puparium formation, an event occurring 48–72h before the onset of diapause, the JH titre, as measured by the *Galleria* wax wound bioassay (de Wilde *et al.*, 1968; de Loof and van de Veire, 1972), is different in diapause and non-diapause destined *Sarcophaga*. In developing individuals, a single pulse of JH may precede the second surge of ecdysone. In diapause destined individuals, JH activity is high at pupariation and major pulses of activity continue at 24h intervals through pupation and into diapause (Walker and Denlinger, 1980; Denlinger *et al.*, 1984). In mid-diapause, JH activity is associated with daily cycles of oxygen

consumption possibly indicating that JH has a role in the regulation of metabolic activity during diapause. The injection of exogenous JH, whilst it will not terminate diapause, will greatly reduce its duration (Fraenkel and Hsiao, 1968b; Zdarek and Denlinger, 1975; Denlinger, 1979).

These two hormones, ecdysone and JH, can interact to break diapause in *Sarcophaga*. Gibbs (1976) noted that the injection of 20-hydroxyecdysone in diapausing *S. argyrostoma* pupae could, if of sufficient dose, cause the initiation of adult development. He further noted that the amount of 20-hydroxyecdysone required to break diapause varied with the age of the pupae. Similar observations were made by Bodnaryk (1977) on the pupal diapause of *Mamestra configurata*. He proposed that diapause could be divided into four different stages depending upon the dose of 20-hydroxyecdysone required to initiate adult development. These stages may yet prove to be applicable to the diapause of *Sarcophaga*. Whilst the injection of JH will not break diapause, it will greatly enhance the effectiveness of injected 20-hydroxyecdysone in terminating diapause (Denlinger, 1979). It is proposed that JH acts by priming the tissues to be targeted by 20-hydroxyecdysone thereby making them more sensitive to stimulation (Denlinger, 1985).

The following investigations will concentrate on the haemolymph ecdysteroid titres, as measured by radioimmunoassay, of diapause and non-diapause destined larvae and pupae of *Sarcophaga argyrostoma*. In addition, a brief determination of the specific ecdysteroids present during prepupal development in both lines will be presented, and the mechanisms of ecdysteroid metabolism in this species will be discussed.

## Materials and Methods

### Culture maintenance

Stock cultures of 'FAST' strain *S. argyrostoma* (see chapter 1) were raised at 25°C under continuous light. The adults were supplied with sugar and water *ad libitum*, and with a daily supply of fresh beef muscle. Newly deposited larvae were transferred on pieces of meat to the supplementary diet described in the general methods section. Fully fed larvae were allowed to wander into dry sawdust to form puparia (Saunders, 1971).

### Larval and pupal ecdysteroid titres in diapause and non-diapause destined *Sarcophaga*

Experimental cultures were established by allowing adult flies to emerge into either LD 12:12 or LD 18:6 at 25°C. Since intrauterine and larval development occurred in these conditions, the former gave a high incidence of pupal diapause (see chapter 1) whilst the latter resulted in its total absence. Meat was supplied on day 4 post eclosion to provide the protein meal required for ovarian development. 24h prior to estimated larviposition on day 12, the meat was removed from the cages followed by the reintroduction of the meat, 24h later, for 2h. This provided a large number of newly deposited larvae all of a similar developmental age. Three cultures of approximately 300 larvae were established in each photoperiod from the appropriate adult cage. The middle of the 2h larviposition period was designated time zero, and all larval time points were measured from this. At time zero, and at 4h intervals thereafter, five larvae were removed from each culture until the onset of pupariation. The

larvae were weighed and examined to determine larval instar and the times of larval moults. In this study, changes in the morphology of the posterior spiracles were used as criteria for the determination of the time of larval moults (figure 3.2) (Saunders, 1986). The growth curve data has already been presented in chapter 1 on larval development, but will be represented here for the sake of clarity.

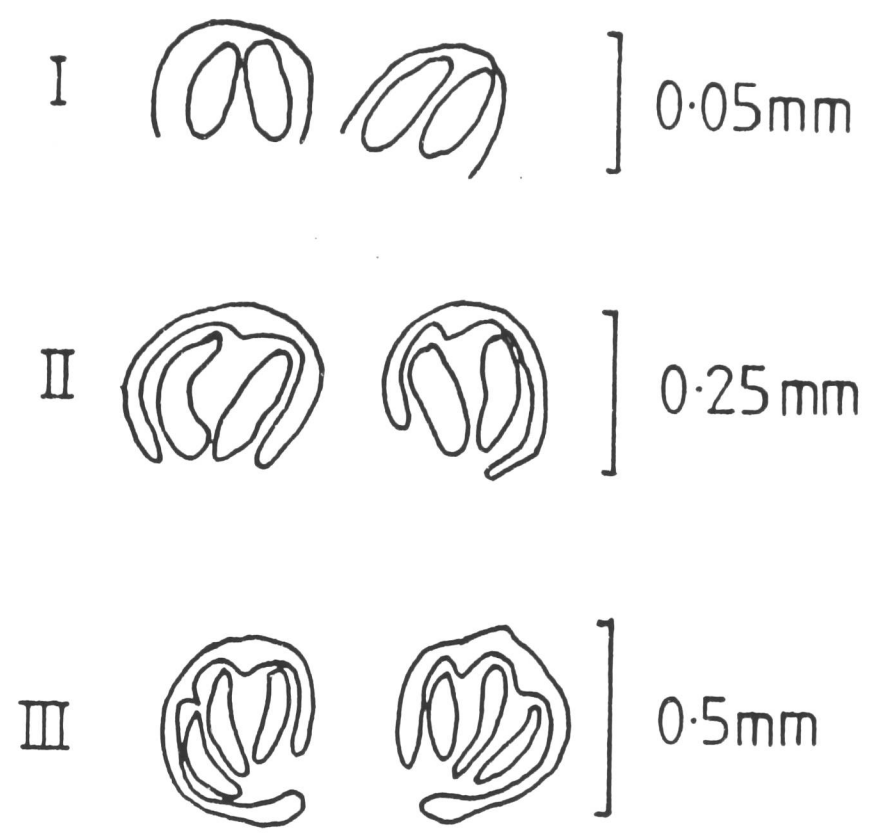


Figure 3.2. The structure of the posterior spiracles of *Sarcophaga argyrostoma* in the three larval instars. After Saunders (1986).

In the first 24h of the investigation, the larvae were too small for haemolymph to be collected, so a pooled, whole body, cold methanol extract was made. The larvae were homogenised in a hand held tissue grinder in 200µl of methanol. Subsequently, a volume of haemolymph, between 10 and 20µl, was taken from each larva and added to nine volumes of cold methanol, the samples being stored at -20°C until analysis. The haemolymph was collected by making a small incision in the posterior of the larva and then squeezing between the fingers to expel the haemolymph onto a clean plastic petri dish. The volume was determined by taking the haemolymph up into the tip of a Gilson P20 Pipetman automatic pipette (set for 20µl), and then turning the dial towards zero until the meniscus reached the end of the tip. The volume could then be read from the dial. A fresh tip was used for each sample.

Following the first major gate of larval exodus, the larvae which had left the food were placed individually in small tubes containing dry sawdust to form puparia without the delaying effects of overcrowding (see chapter 1). These newly formed puparia were collected at 4h intervals, and subsequent puparial samples timed from this point. As pupal and pharate adult development commenced, the haemolymph became contaminated with large quantities of dissociated fat body cells which were removed by centrifugation prior to analysis by radioimmunoassay.

The diapause incidences of the long day and short day lines were checked ten days after the formation of the puparia, as described in the general methods section.

#### **Haemolymph ecdysteroid analysis**



Prior to analysis, the extracts were centrifuged at 13000xg for 5 minutes to remove any cellular debris and precipitated proteins. Aliquots were taken from the resultant supernatant. The haemolymph ecdysteroid titres were then determined on two aliquots of larval (25µl) or pupal (20µl) haemolymph extracts by radioimmunoassay (RIA) employing an antiserum (H-22) that bound ecdysone and 20-hydroxyecdysone in a ratio of 1:1.9, but was not useful for measuring side-chain oxidised or conjugated ecdysteroid metabolites (Gilbert *et al.*, 1977). Five extracts were assayed for each haemolymph concentration. [<sup>3</sup>H] ecdysone (60 Ci/mmol New England Nuclear) was used as the tracer and titres were expressed in ecdysone equivalents. The antibody-bound fraction was precipitated by a second antibody (donkey anti-rabbit IgG, Scottish Antibody Production Unit) and counted in Ultrafluor scintillation fluid (National Diagnostics) in a Packard 2425 Tricarb liquid scintillation counter (counting efficiency 36.5%). The full method is described in the radioimmunoassay methodology appendix.

### **Specific ecdysteroid analysis**

#### **a) Sample preparation**

Haemolymph samples from 4, 8, and 12h puparia, either diapause or non-diapause destined, were pooled and extracted with three volumes of methanol. Following centrifugation, the residues were further extracted with 50% aqueous methanol, and partitioned against hexane to remove non-polar lipids. The diapause and non-diapause samples were evaporated and taken up in high performance liquid chromatography buffer (HPLC) (2.4ml, 20mM TRIS/perchlorate, pH 7.2) and applied to primed C18 Sep Paks (Waters). These are in effect small reverse phase chromatography columns and were primed with 10ml of methanol, to expel any air, 10ml of distilled water and 10ml of HPLC

buffer. The sample was injected onto the Sep Pak slowly to allow maximum retention of ecdysteroid material. After a 2ml buffer wash, the ecdysteroids were eluted with 7ml of methanol. The methanol was then evaporated, the residues dissolved in buffer (1ml) and the samples centrifuged to remove any particulate matter immediately prior to HPLC analysis.

#### b) HPLC conditions.

A Waters gradient elution HPLC system (two 6000A pumps, 720/730 system integrator and controller, Schoeffel UV detector (242nm) and an LKB fraction collector) was employed for ion suppression, reverse phase chromatography (IS-RPHPLC) using a 5 $\mu$ m Resolve (Waters) C18 column (15cm x 4.6mmID). The solvent system for gradient elution was: Solvent A (5% acetonitrile, 95% 20mM TRIS/perchlorate buffer, pH 7.5), Solvent B (5% buffer, 95% acetonitrile); conditions as indicated in the results section, figures 3.6 and 3.7 (see Warren and Gilbert, 1986, for details). Fractions (0.5ml) were collected from which duplicate aliquots (20ul) were assayed for ecdysteroid immunoreactivity. Two complimentary antisera were employed in the analyses. One (H-22), raised against an ecdysone-22-succinyl-thyroglobulin immunogen, detects ecdysone and its metabolites resulting from phase 1 (oxidation) and phase 2 (conjugation) reactions of the side chain; and H-2 (derived from a 20-hydroxyecdysone-2-succinyl thyroglobulin immunogen) capable of detecting phase 1 and phase 2 metabolites of the A-ring of ecdysone, e.g., 3- $\alpha$ -epimers and ester conjugates at C-2 and C-3 (Warren and Gilbert, 1986). Assay composition and termination by protein A are described fully in the appendix on RIA methodology.

#### c) Ecdysteroid hydrolysis

Following IS-RPHPLC, fractions containing highly polar products and conjugates (HPP, 0–35 minutes) from both long and short day lines were pooled, evaporated (reduced pressure at 35°C) and the residue taken up in 0.05M acetate buffer, pH 5.0 (10ml), containing 10mg each of lyophilised crude esterases from *Helix pomatia* (Sigma H-1) and acid phosphatase from potatoes (Sigma). Following incubation overnight at 37°C, the centrifuged solution (together with a buffer wash of the precipitate) was applied to a primed C18 Sep Pak which was washed (2ml HPLC buffer) and eluted (7ml methanol) as before, prior to analysis by IS-RPHPLC.

## Results

The rates of growth were similar in both the long and short-day cultures (fig. 3.3). Both cultures showed a thousand fold increase in weight over the 72h period following larviposition. Subsequent post-feeding weight loss by the long day culture was more rapid than that of the short day culture, but following larval exodus, those larvae raised under LD 12:12 conditions wandered for 24h longer than those under LD 18:6. The two cultures reached the same mean larval weight by the onset of pupariation. The diapause incidences of the long and short day cultures were 0 and 89% respectively.

Figures 3.4A and 3.4B show the haemolymph ecdysteroid titres obtained under the two photoperiods. Both cultures displayed a high, whole body ecdysteroid titre at larviposition (550 pg/ $\mu$ l and 1220 pg/ $\mu$ l respectively), and haemolymph peaks prior to each moult (between 200 and 300pg/ $\mu$ l). Another large ecdysteroid surge (250pg/ $\mu$ l) occurred before larval exodus from the food. The two cultures exhibited similar titres except that those larvae raised under LD 18:6 had a higher mean post feeding titre ( $97 \pm 21$  pg/ $\mu$ l) than those raised under LD 12:12 ( $48 \pm 8$  pg/ $\mu$ l), as determined for the period up to the onset of pupariation. The profiles between 96 and 124h are very similar in both lines. The mean titre of the short day culture was  $57 \pm 8$  pg/ $\mu$ l and that of the long day line was  $65 \pm 13$  pg/ $\mu$ l during this period. Following this elevated titre, both cultures display a peak at 135h ( $\approx 200$  pg/ $\mu$ l in LD 18:6;  $\approx 120$  pg/ $\mu$ l in LD 12:12). This large peak immediately preceded puparium formation only in the long day culture (Fig 3.4B). The peak in the short day culture did not appear to be coincident with the onset of pupariation (Fig 3.4A).

Figures 3.5A and 3.5B show the prepupal, pupal, and pharate adult

ecdysteroid titres in diapause and non-diapause induced cultures, timed from the formation of the puparium. Both cultures showed a high titre ( $\approx 500$  pg/ $\mu$ l) following pupariation, presumably associated with larval/pupal apolysis, and a gradual drop through 24 and 48h. The titre in the diapause destined culture remained low except for a small peak ( $\approx 100$  pg/ $\mu$ l) at 76h, whereas that in the developing culture rose to twice that level ( $\approx 200$  pg/ $\mu$ l). This peak may be involved with pupal/adult apolysis and the initiation of pharate adult development in the non-diapause destined culture. In the short-day culture, this peak may be the result of diapause not being saturated (*i.e.* 89 rather than 100%) under these conditions since it is not possible to distinguish diapausing and non-diapausing pupae at this age. The actual titres of the five individual pupae sampled at this time point were 46, 287, 12, 262, and 28 pg/ $\mu$ l which gave a mean  $\pm$  SEM of  $127 \pm 60$ . Two values were considerably higher than the others suggesting that some developing pupae were sampled along with the diapausing pupae. Following this stage, non-diapausing pupae could be removed from the samples prior to the ecdysteroid assay. The most prominent difference between the two cultures is seen after 80h. The developing insect titre is characterised by a large peak (550 pg/ $\mu$ l) at 85–90h which is presumably associated with unspecified developmental events in the pharate adult. Following this peak, the titre fell slowly over the remainder of experimental period. Other peaks may have been present but not detected because of the less rigorous sampling protocol employed after 100h. The titre in the short day culture however, fell to a level close to the lower limit of detection of the radioimmunoassay used in this investigation as the pupae entered and remained in diapause.

Few qualitative differences were observed among the haemolymph ecdysteroids in diapause and non-diapause destined *Sarcophaga* prepupae.

Figures 3.6 and 3.7 show the haemolymph ecdysteroid profile of prepupae raised under LD 18:6 and LD 12:12 respectively. The two most prominent peaks in both lines correspond to 20-hydroxyecdysone and ecdysone, respectively. The areas of RIA positive activity in the H-22 traces between fractions 20 and 70 correspond to a series of poorly resolved highly polar products, that may represent side-chain ester conjugates and ecdysonic acids. These fractions account for approximately 60% of the RIA activity in both diapause and non-diapause destined lines. The upper traces support the identification of these ecdysteroids by the sensitivity of the H-2 antiserum to 20-hydroxyecdysone and ecdysone, but not to the other components, thereby ruling out 3- $\alpha$ -epimers or A-ring conjugates. The concentrations of the free ecdysteroids from diapause and non-diapause destined lines are shown in Table 3.1. No major differences between the levels of each ecdysteroid identified were observed. These ecdysteroids have not been identified unequivocally because the data are based on analyses by reverse phase HPLC only. Absolute identification would require further analysis by normal phase HPLC or preferably by mass spectroscopy.

Figure 3.8 shows the ecdysteroid profile of the hydrolysed HPP fractions from the pooled LD 18:6 and LD 12:12 samples. The levels of conjugated ecdysone and 20-hydroxyecdysone are much lower than in the free ecdysteroid fractions (Table 3.2). The conjugated levels of 20,26-dihydroxyecdysone are approximately five times higher than the free levels, and the levels of 26-hydroxyecdysone are forty times higher. A significant level of makisterone A was noted in the HPP conjugate fraction. The position of the highly polar 20-hydroxyecdysoneic acid did not change after the deconjugation procedures which suggested that it was not subject to any conjugation.

It would appear that the overall levels of ecdysteroids in the diapause and non-diapause destined lines of *Sarcophaga* are similar, and that no significant differences were noted in the levels of specific detectable ecdysteroids.

Table 3.1. The levels of free specific ecdysteroids detected in diapause (LD 12:12) and non-diapause (LD 18:6) destined pupae of *Sarcophaga argyrostoma* corrected for cross reactivity (CR) with H-22 antiserum. Concentration in ng/ml of haemolymph.

Ecdysteroid	CR	LD 18:6	LD 12:12
Ecdysone	1	41	29
20-hydroxyecdysone	4.5	263	237
20,26-dihydroxyecdysone	5	34	38
Makisterone A	5	-	8
Highly Polar Products	1	129	144



Table 3.2. The levels of conjugated ecdysteroids and ecdysonoic acids extracted from the pooled highly polar fractions of 4,8, and 12h post-pupariation haemolymph from diapause and non-diapause destined pupae of *Sarcophaga argyrostoma*. Results corrected for cross reactivity (CR) with H- 22 antiserum. C/F= Ratio of conjugated ecdysteroid to free ecdysteroid. Concentrations in ng/ml of haemolymph.

Ecdysteroid	CR	Conc	C/F
Ecdysone	1	12	0.34
20-hydroxyecdysone	4.5	40	0.16
26-hydroxyecdysone	1	38	38
20,26-dihydroxyecdysone	5	186	5.2
Makisterone A	5	108	27
Ecdysonoic acid	1	7	0
20-hydroxyecdsonoic acid	5	254	0

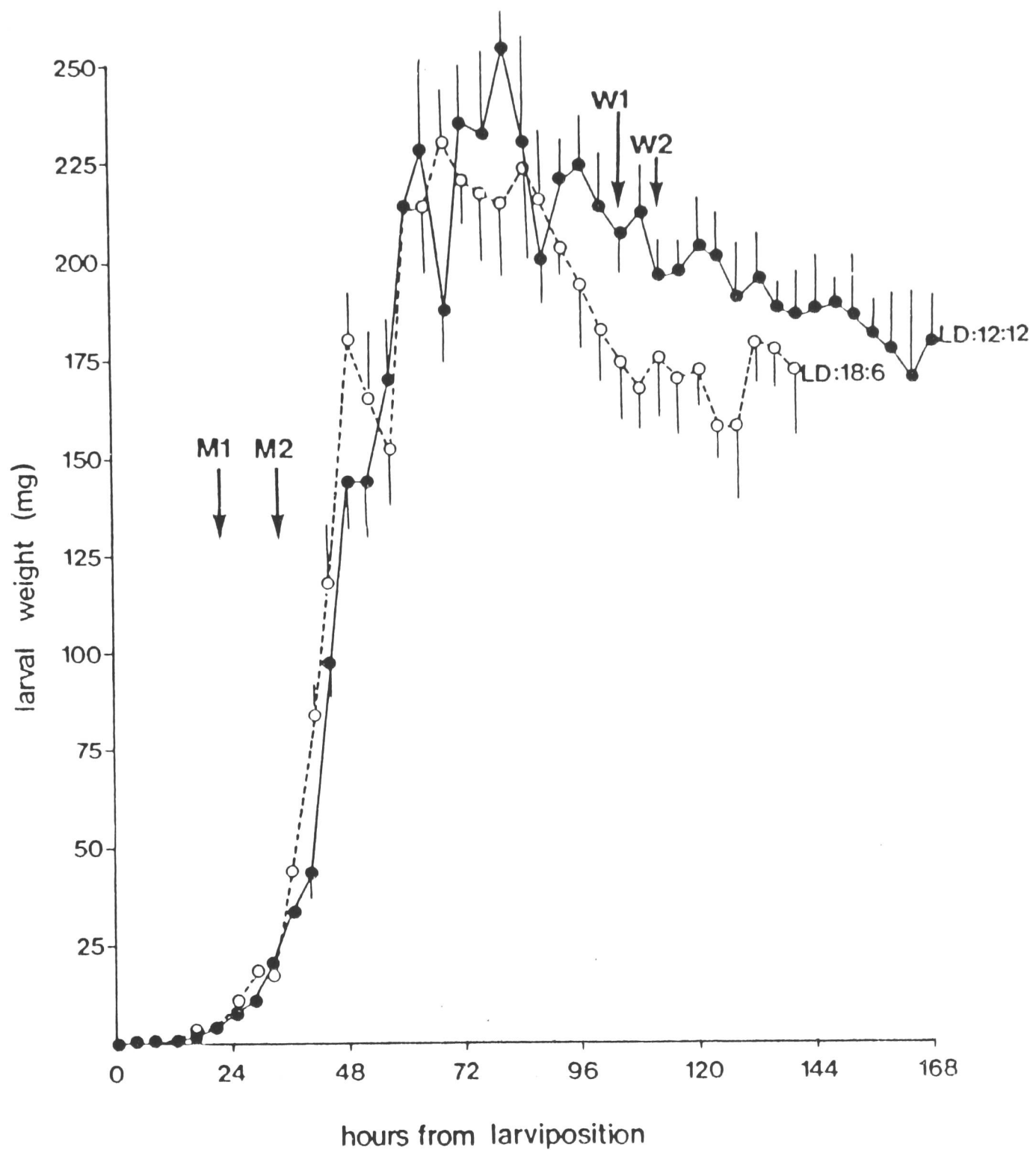


Figure 3.3. The growth and development of larval *Sarcophaga argyrostoma* maintained in either LD 12:12 (●---●) or LD 18:6 (○---○) conditions at 25°C. Each point represents the mean of five weighings  $\pm 2SE$ . M1 and M2 denote the first and second larval moults, respectively. W1 and W2 denote the onset of larval wandering from the food for LD 18:6 and LD 12:12, respectively.

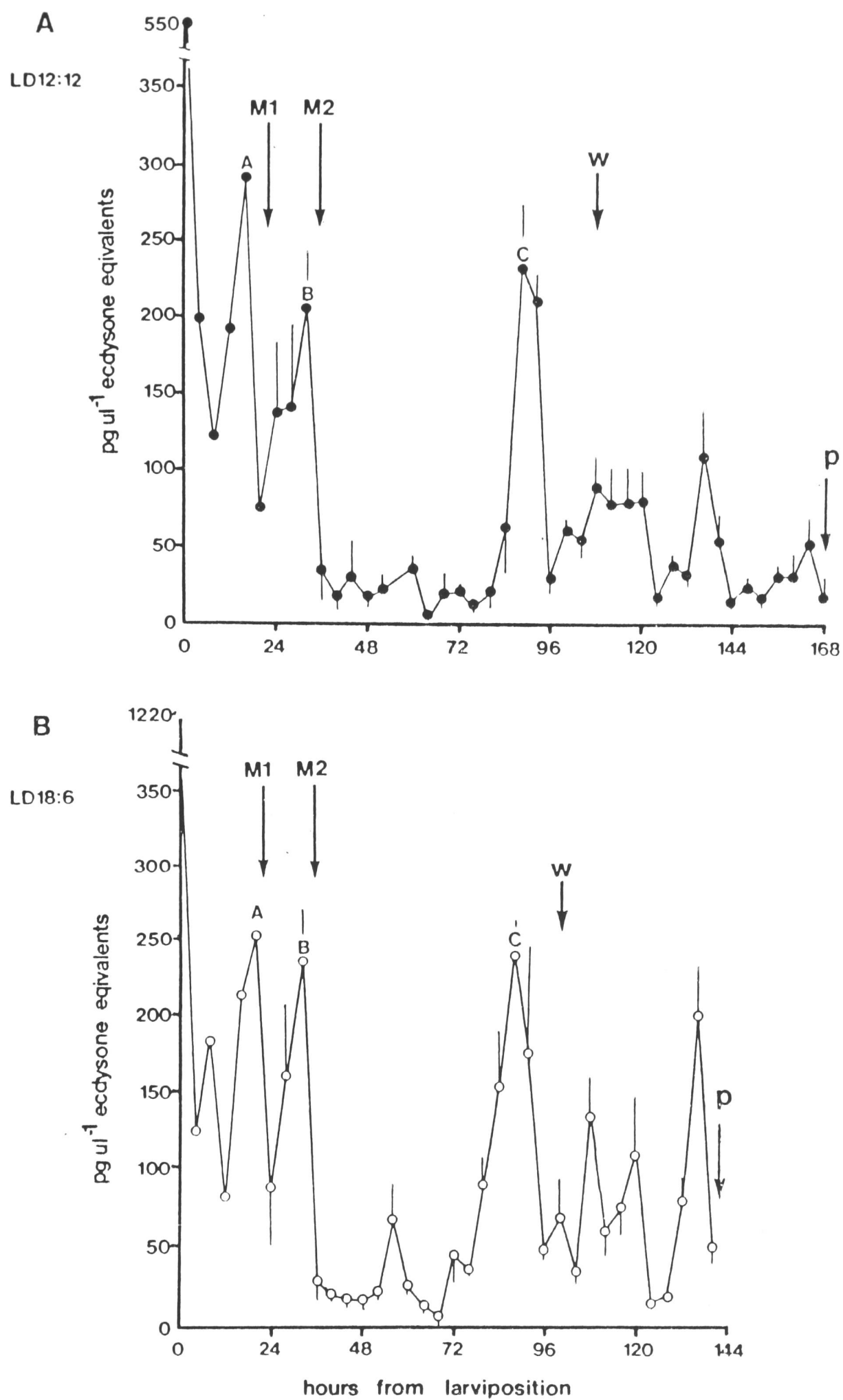


Figure 3.4. The ecdysteroid titres of larval haemolymph from cultures raised under A. LD 12:12 or B. LD 18:6. A,B and C are peaks associated with larval moults (M1 and M2) and the onset of larval wandering (W). P is the onset of pupariation. Data in the first 24h of larval development are based on pooled whole body homogenates. Subsequent points represent the mean of five determinations  $\pm$  SEM.

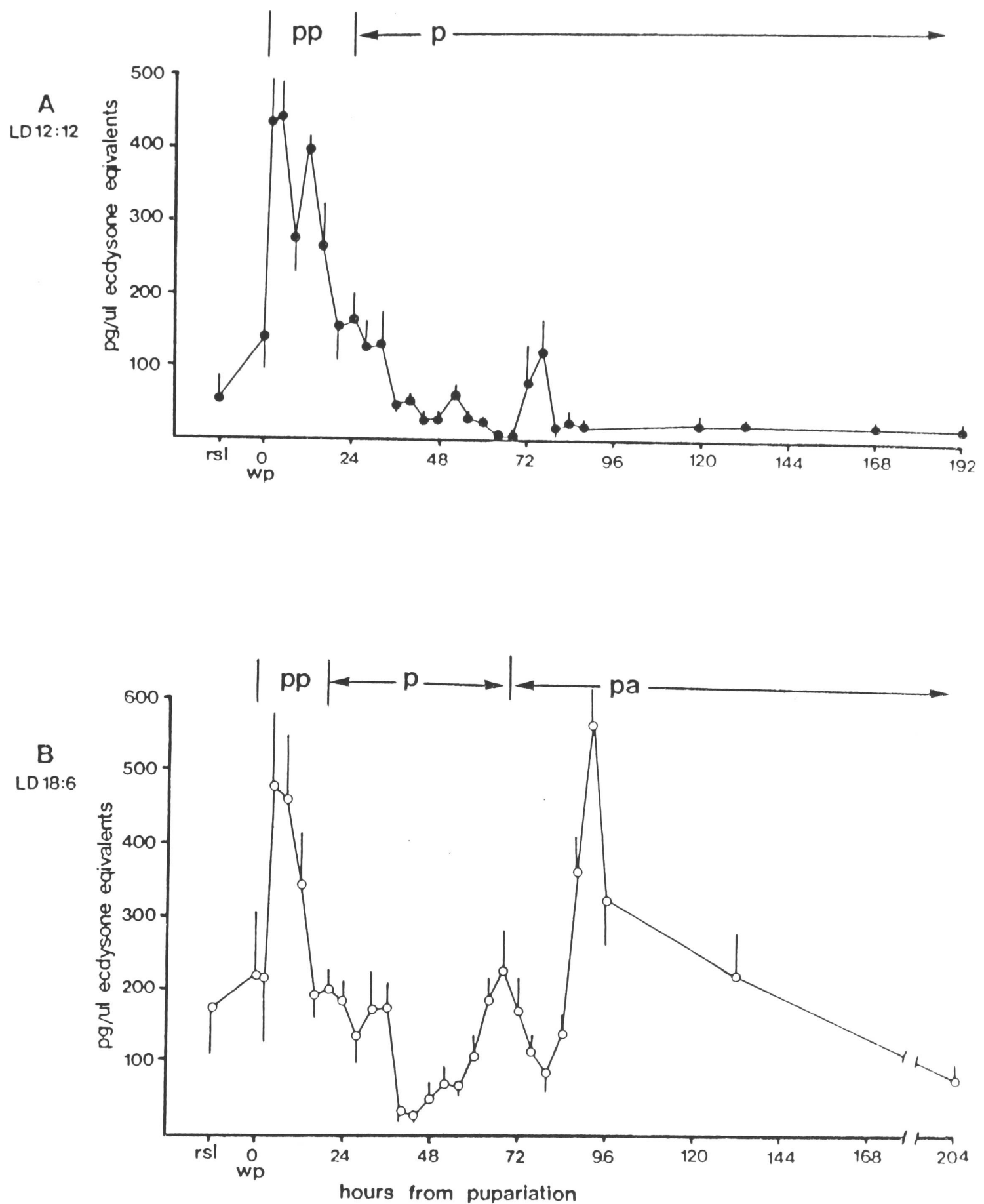


Figure 3.5. Pupal haemolymph ecdysteroid titres under (A) diapause inductive and (B) non-diapause inductive photoperiods at 25°C. Each point represents the mean of five determinations  $\pm$ SE. RSL, red spiracled larvae; WP, white puparia; PP, prepupal; P, pupal; PA, pharate adult.

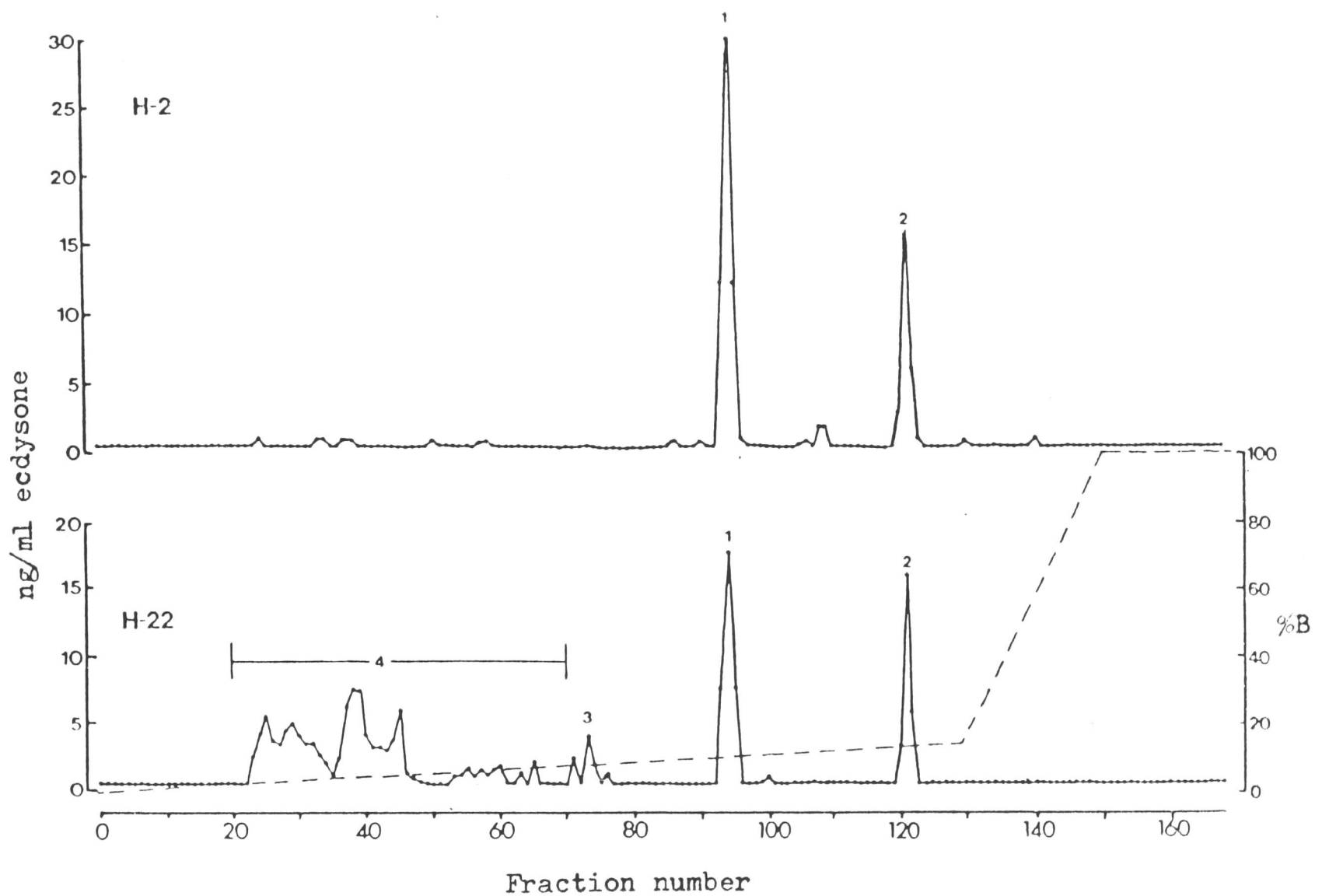


Figure 3.6. IS-RPHPLC trace of free ecdysteroids extracted from pooled haemolymph samples from 4,8, and 12h non-diapause destined puparia. 1. 20-hydroxyecdysone, 2. ecdysone, 3. 20,26-dihydroxyecdysone, 4. highly polar ecdysteroid products. Ecdysteroids quantified by radioimmunoassay (RIA) on collected fractions with H-2 (top trace) and H-22 (bottom trace) antisera against an ecdysone standard curve. The dashed line shows the gradient elution profile of the analysis. Solvent A, 95% 20mM TRIS perchlorate buffer pH 7.2, 5% acetonitrile. Solvent B, 5% TRIS perchlorate buffer, 95% acetonitrile. Waters 15cm x 4.6mm ID 5um ODS column. Flow rate 1ml/minute.

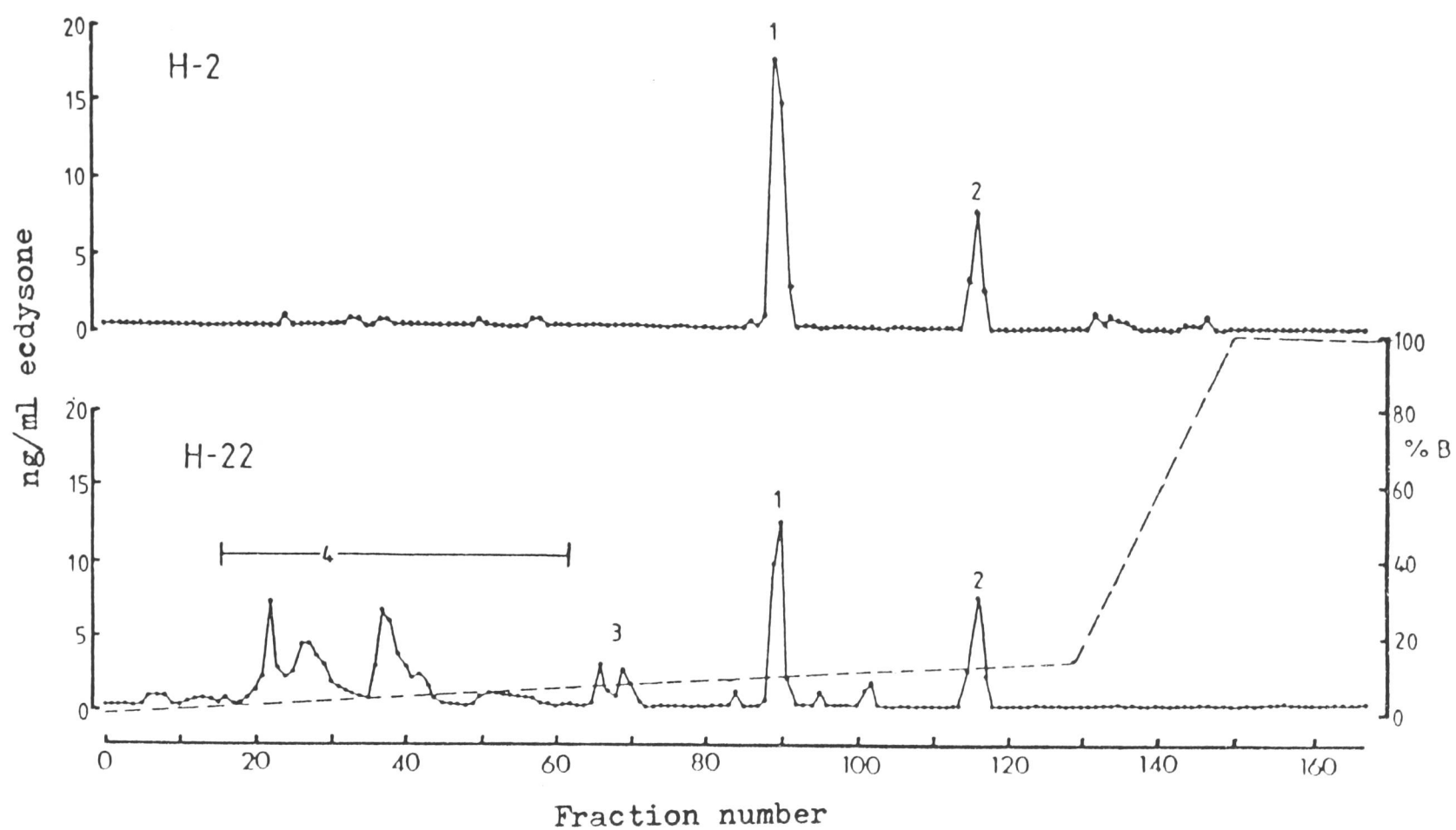


Figure 3.7 IS-RPHPLC trace of free ecdysteroids extracted from pooled haemolymph samples from 4,8, and 12h diapause destined puparia. 1. 20-hydroxyecdysone, 2. ecdysone, 3. 20,26-dihydroxyecdysone, 4. highly polar ecdysteroid products. Ecdysteroids quantified by radioimmunoassay (RIA) on collected fractions with H-2 (top trace) and H-22 (bottom trace) antisera against an ecdysone standard curve. The dashed line shows the gradient elution profile of the analysis. Solvent A, 95% 20mM TRIS perchlorate buffer pH 7.2, 5% acetonitrile. Solvent B, 5% TRIS perchlorate buffer, 95% acetonitrile. Waters 15cm x 4.6mm ID 5µm ODS column. Flow rate 1ml/minute.

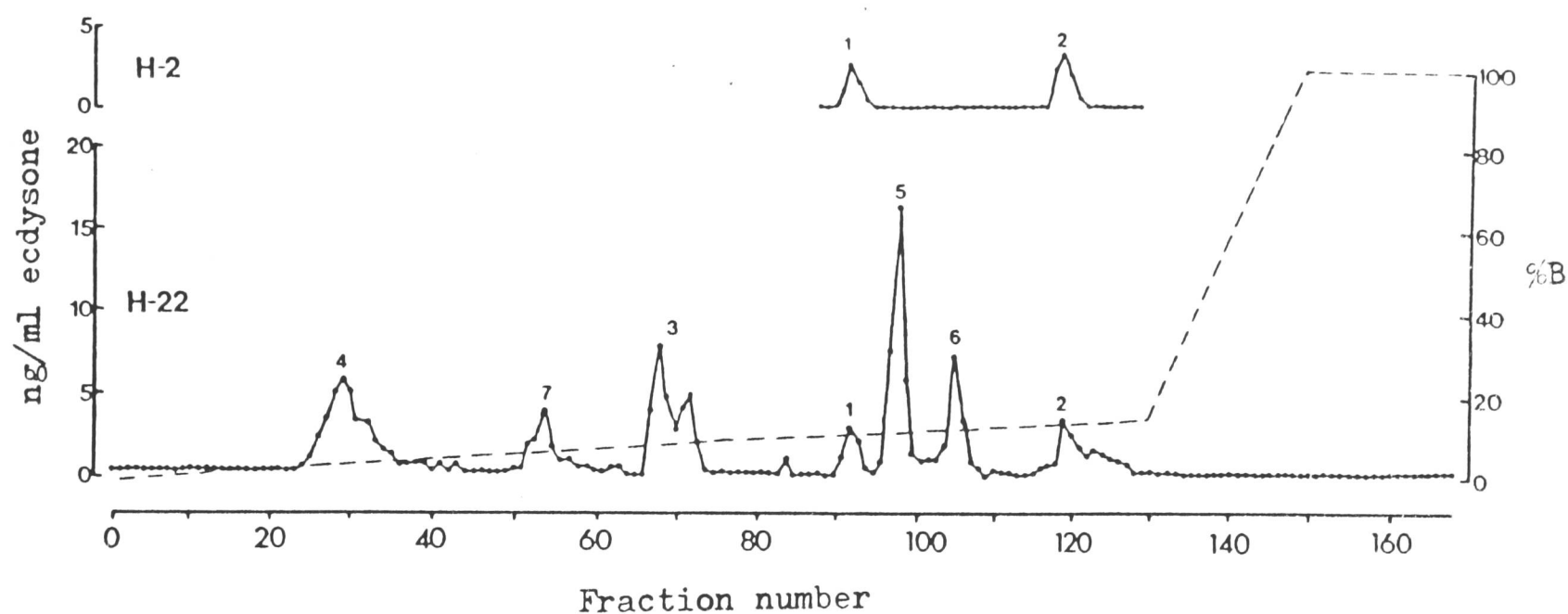


Figure 3.8. IS-RPHPLC trace (H-22 antiserum) of ecdysteroids pooled from highly polar products isolated from initial HPLC separations of diapause and non-diapause destined pupal extracts following deconjugation by a mixture of crude esterases. 1, 20-hydroxyecdysone, 2. ecdysone, 3. 20,26-dihydroxyecdysone, 4. 20-hydroxyecdysoneic acid, 5. 26-hydroxyecdysone, 6. makisterone A, 7. ecdysoneic acid. IS-RPHPLC and RIA conditions as for figures 3.5 and 3.6.

## Discussion

The development and moulting of *Sarcophaga argyrostoma* is controlled by the endocrine system. A moult occurs in response to a pulse of 20-hydroxyecdysone in the haemolymph, although the precise mechanism of its action is not fully understood. The two larval/larval moults typical of the Sarcophagidae were noted 24 and 48h after larviposition at 25°C. These times are similar to those reported by Roberts (1976) for the Australian flesh-fly *Tricholioproctia impatiens*, and by Wentworth *et al.* (1981) in *S. bullata*. There appears to be little difference between those lines raised under diapause and non-diapause inducing conditions. Following larval exodus from the food however, those larvae raised under short day conditions wandered for 24h longer than those raised under long day conditions. It was demonstrated in chapters 1 and 2 that pupariation within a population occurs over several days without any apparent circadian influence, and that those cultures raised under long day photoperiods pupariated over a lesser time than those raised under short days. The mean weight of the populations was approximately the same in each photoperiod at the onset of pupariation, possibly indicating that under these nutritional conditions, a particular proportional weight loss, or a particular eventual size (less likely) may influence the release of PTTH causing the formation of the puparium.

The larval ecdysteroid titres reported here are characterised by a high level at larviposition, followed by a rapid drop by 12h. Unlike the majority of the titres reported here, these were measured in whole body extracts because of the difficulty of obtaining haemolymph from such small larvae. These very high



titres have not previously been described for newly deposited *Sarcophaga* larvae . Wentworth *et al.* (1981) reported that at larviposition, the whole body titre of *S. bullata* was approximately 10 pg/mg fresh weight. Similar low titres were found in adult ovaries prior to larviposition (Wentworth *et al.*,1984). Sall (1982) however, demonstrated that various ecdysteroids can accumulate in the embryos of *Locusta migratoria* and Imboden *et al.* (1978) showed high levels of 20-hydroxyecdysone in the haemolymph of the embryonic cockroach *Nauphoeta cinerea* . The high titres in this investigation may therefore have resulted from similar ecdysteroid accumulations associated with intrauterine larval development and possibly with hatching from the chorion which normally occurs within the female abdomen. The high levels of ecdysteroids in embryos may be excreted soon after larviposition. Since larvae were collected at the earliest possible moment after normal larviposition, such ecdysteroids may have been detected. The difference between the titres in the two photoperiods at this stage may be due to slight differences in the time of sampling which, considering the very rapid fall in the whole body ecdysteroid level have resulted in what is a large variation. Alternatively, a factor or factors may have been present in the extracts around the time of larviposition which interfered with the radioimmunoassay. Such a factor may not have been present in the *S. bullata* studies, possibly due to dietary differences. In the present study, stock larvae were fed on beef muscle and a yeast/milk powder supplement, and adults on sugar and water, with meat supplied to promote embryonic growth, whereas in the *S. bullata* studies (Wentworth *et al.*,1981,1984), larvae were fed exclusively on lamb liver, and adults on a mixture of sugar and wheatgerm with water and a liver protein source. To test whether the high titres reported in the present study are genuine, it would be necessary to follow the whole body ecdysteroid titres of embryos from the

appearance of a suitable synchronizing feature, possibly the appearance of spiracles or the initiation of movement (see chapter 1), through to early larval development. The identification of ecdysteroids present by HPLC would confirm whether an accumulation does indeed occur before larviposition. The presence and role of ecdysteroids in embryonic development in insects is reviewed by Hoffman and Lagueux (1985).

Prior to each of the two larval moults, a pulse of ecdysteroids is seen. After these ecdyses, the titre remains low in both lines until just before the onset of wandering behaviour when a large pulse of ecdysteroid release occurs. Dominick and Truman (1984) have shown that the onset of wandering in *Manduca sexta* is a circadian controlled (gated) event to which the larvae are committed by a release of prothoracicotropic hormone (PTTH), and the accompanying elevation of ecdysteroid levels (Gilbert *et al.*, 1981). Chapter 2 has shown that the onset of wandering behaviour in *S. argyrostoma* is also a gated event. Presumably therefore, the increase in haemolymph ecdysteroid titre reported here is in response to an earlier gated release of PTTH by the brain. This wandering peak may also be involved in changing the commitment of the tissues from larval to pupal syntheses. Riddiford (1976) demonstrated that this peak, the first to occur in the absence of JH, programmed the epidermal cells of *M. sexta* to synthesize pupal, rather than larval, cuticle in response to the next major peak of haemolymph ecdysteroids. Whilst this wandering peak is gated in both *Sarcophaga* (chapter 2), and *Manduca* (Dominick and Truman, 1984), there is little documented evidence that ecdysone release prior to larval moulting is gated.

Following wandering, the levels of circulating ecdysteroids are variable. The similar titres between the two lines from 96 to 124h may be involved in the

preparation of the larvae for later development. The process of pupariation may require a later synthesis of ecdysone which was not detected in both lines. The higher average titre over the whole wandering period in the non-diapause destined cultures may be associated with the reduction of the period, in that a higher titre may encourage earlier puparium formation. A development destined individual will only require a pupation site for approximately 12 days, whereas a diapause destined one will require the site for several months during a time of adverse environmental conditions, so a more thorough 'selection' of the site may optimise the chances of survival (Denlinger, 1972). It is possible that this higher titre may be either a cause or an effect of the rate or proportion of weight loss after wandering. Doane (1972) suggested that PTTH release may be influenced by nutritional state. The involvement of internal cues acting via proprioceptors has also been proposed (Gilbert *et al.*, 1980). These two factors may interact to partially control the timing of PTTH release, although environmental factors such as the availability of a suitable pupariation site may also have a controlling influence. Ohtaki (1966) reported that the larvae of *S. peregrina* do not pupariate in a wet condition unless they have previously been exposed to a dry situation for a certain period. In the present experiments, those factors which can delay pupariation (crowding and the lack of a suitable site) were not present, thereby allowing other factors, presumably the result of previous photoperiodic experience, to influence development.

Wentworth *et al.* (1981) showed the presence of a large ecdysteroid peak prior to puparium formation in *S. bullata*. Due to developing asynchrony within the short day culture by this stage in the present investigation, it was only possible to show the existence of this peak in the long day larvae. Unlike the onset of wandering, puparium formation is not a gated event in *S.*

*argyrostoma* (See chapter 2), although Roberts (1984) did suggest a weak association with the dark phase in *S. bullata*. In the present investigation therefore, puparia were collected every four hours to ensure the synchrony of the timings of the puparial ecdysteroid samples. There were no observed differences in these ecdysteroid titres between the two lines in the first 48h of intrapupal development. Both cultures displayed a large peak in the first 24h of prepupal/pupal development associated with larval/pupal apolysis (Wentworth *et al.*, 1981). Following pupariation, the titres gradually drop below 100 pg/ul after 48h. The peak noted around 72–76h may be associated with the initiation of pupal/adult apolysis (Roberts and Warren, 1975) and may elicit the onset of pharate adult development. The titres reported in the present study agree with those described by Briers *et al.* (1983) in a number of Dipteran species. There would appear therefore to be a degree of uniformity in the endocrine control of developmental events at the prepupal/pupal stage. The lower ecdysteroid level in the diapause destined culture may not be enough to bring about such development. Alternatively, the peak at 72h may be the result of the unsaturated diapause incidence reported in this investigation. The raw data shows that two samples contained levels of ecdysteroids similar to those found in the developing culture whereas the others were very low. It was not possible to remove those pupae which were not entering diapause from the samples at this stage of development. Once pharate adult development had progressed for another 24h it was possible to recognise those which were not entering diapause and remove them from the sampling regime. The haemolymph titre in the short day individuals remained very low as they entered diapause, whereas the level of circulating ecdysteroids in the long day insects increased between 85 and 90h to a very high level (600 pg/ul), as pharate adult development commenced. Similar titres

were reported by Walker and Denlinger (1980) in *S. crassipalpis* and by Ohtaki and Takahashi (1972) in *S. peregrina* using a *Musca* bioassay technique. Both these groups reported the absence of the adult development peak of ecdysteroids in diapausing flesh flies. The timing of the endocrinological shutdown in diapausing pupae of *S. argyrostoma* is consistent with that reported by these workers and by Roberts and Warren (1975) in *T. impatiens*.

These results support the theory that diapause results from the failure of the brain/prothoracic gland system to effect the production of ecdysone. This is further supported by the observation that diapause in *Sarcophaga* can be terminated by the injection of 20-hydroxyecdysone into diapausing pupae (Gibbs, 1976; Zdarek and Denlinger, 1975). That Fraenkel and Hsiao (1968b) were able to break diapause by the injection of ecdysone, the prohormone to 20-hydroxyecdysone, implies that the enzymatic systems responsible for ecdysone hydroxylation, usually located in the fat body (Gilbert *et al.*, 1980), are either competent to respond, or easily switched on. Bowen *et al.* (1984a) further demonstrated that prothoracic glands from the diapausing pupae of *Manduca sexta* were not as competent to respond to PTTH stimulation *in vitro* as glands from non-diapausing pupae. The relevance of this observation to pupal diapause in *Sarcophaga* and larval diapause in *Calliphora vicina* will be discussed in chapter 4. It is not possible to relate the observations of Walker and Denlinger (1980) and Denlinger *et al.* (1984), that juvenile hormone titres fluctuate during diapause in *S. crassipalpis*, to diapause in *S. argyrostoma* since no investigations into JH titres in this species have been conducted. However, these cyclic fluctuations may indeed exist as a regulatory mechanism of metabolism during diapause.

The levels of specific ecdysteroids in the haemolymph of diapause and

non- diapause destined prepupae show few differences between the two lines. Both contain ecdysone, 20-hydroxyecdysone and 20,26-dihydroxyecdysone as the major free ecdysteroids. The highly polar 20-hydroxyecdysoneic acid appears not to have been conjugated as it eluted at the same position after the deconjugation procedures. Other putative free ecdysteroids noted were 26-hydroxyecdysone and makisterone A, although at much lower levels. The presence of the 28 carbon makisterone A in the free ecdysteroid titre suggests that the diet supplied to the developing larvae contained a suitable substrate for the production of this steroid. Redfern (1984) demonstrated that under suitable dietary conditions, *Drosophila* could produce 2-deoxymakisterone A as well as more normal 27 carbon ecdysteroids. Data presented here suggest that this may also be the case with *Sarcophaga argyrostoma*.

The levels of conjugated ecdysteroids determined after enzymatic hydrolysis indicate that conjugation is an important pathway for ecdysone catabolism in *Sarcophaga*. It is generally accepted that ecdysone is hydroxylated by ecdysone 20-monooxygenase to the active form, 20-hydroxyecdysone (Bollenbacher *et al.*, 1977). The site of this hydroxylation is apparently not restricted to only one tissue . King (1969,1972a,1972b) demonstrated that the major sources of the enzyme responsible for this reaction, ecdysone-20-monooxygenase, were the fat body, the Malphigian tubules, the midgut, integument and possibly the gonads. Bergamasco and Horn (1980) noted that additional and/or alternative hydroxylation at C-26, converting ecdysone to 26-hydroxyecdysone and 20-hydroxyecdysone to 20,26-dihydroxyecdysone, reduces the biological activity of the hormone. Both of these metabolites are found in significant quantities in the conjugate fraction indicating the presence of a 26- monooxygenase, and the subsequent conjugation of these 26-hydroxylated metabolites. No evidence was found of

the presence of 3- $\alpha$ -epimers or A- ring conjugates in the conjugate fraction. It would appear that under these conditions, *Sarcophaga* either cannot or does not effect these deactivation mechanisms in ecdysone metabolism. The major conjugation site in this species would therefore appear to be C-22 on the steroid side-chain (See figure 3.1).

26-hydroxyecdysteroids can be further oxidised to the corresponding carboxylic acids (Koolman,1982). 26-hydroxyecdysone may be converted directly to ecdysonic acid which can then be 20-hydroxylated to 20-hydroxyecdysonic acid. Alternatively, 26-hydroxyecdysone may be first 20-hydroxylated and then oxidised to 20-hydroxyecdysonic acid. That little ecdysonic acid was found suggests that the latter route is preferred in *Sarcophaga*. In addition, 20,26-dihydroxyecdysone produced by the 26-hydroxylation of 20-hydroxyecdysone, may also be 26-oxidised to 20-hydroxyecdysonic acid.

The results show that 26-hydroxyecdysone, 20,26-dihydroxyecdysone and makisterone A are preferentially conjugated over ecdysone and 20-hydroxyecdysone which exist predominantly in the free form. It would appear that the predominant metabolic pathway for ecdysone is hydroxylation to 20- hydroxyecdysone and 26-hydroxyecdysone, the latter then being preferentially conjugated. 20-hydroxyecdysone appears to be C26- hydroxylated followed by C26- oxidation to 20-hydroxyecdysonic acid. It is not possible to make any conclusions as to the mechanisms of conjugation involved in the metabolism of ecdysone in *Sarcophaga* because a mixture of hydrolysing enzymes was used in this investigation. Nor is it possible to speculate upon the presence of storage conjugates as proposed by Koolman (1985) since no information as to the form of the conjugates is available.

The absence of any observed differences in specific ecdysteroid titres between diapause and non-diapause destined lines supports the suggestion that the regulation of diapause induction is probably centred in the brain (Bowen *et al.*, 1985). The most likely system controlling diapause is the regulation of the release of prothoracicotropic hormone, although secondary downstream blocks involving prothoracic gland competency are possible (Bowen *et al.*, 1984). The involvement of these two factors on the induction and termination of diapause in both *Sarcophaga* and *Calliphora* will be investigated in chapter 4.



## CHAPTER 4

Prothoracic gland function in diapause and  
non-diapause destined *Sarcophaga argyrostoma*  
and *Calliphora vicina*

**Prothoracic gland function in diapause and non-diapause  
destined *Sarcophaga argyrostoma* and *Calliphora vicina***

### Introduction

The endocrine function of the insect brain was first suggested by Kopec (1922) who postulated the existence of a brain hormone in the gypsy moth *Lymantria dispar* which was necessary for stimulating the moulting process. This hormone, now termed the prothoracicotropic hormone (PTTH) has been shown to stimulate the prothoracic glands to produce ecdysone, the prohormone to 20-hydroxyecdysone, which effects moulting. This chapter will concentrate upon the action of PTTH extracted from the prepupal brains of *Sarcophaga argyrostoma* on the prothoracic glands of both *S. argyrostoma* and *Calliphora vicina in vitro*. The mechanism of PTTH action in these species will be investigated and the role of the brain/prothoracic gland axis in diapause induction and termination will be discussed.

PTTH in the tobacco hornworm *Manduca sexta* has been shown to be a cerebral neuropeptide which exists in two molecular forms (Bollenbacher *et al.*, 1984). Using gel filtration chromatography, these workers determined that PTTH activity was found in the 29 kD (Molecular weight=29,000) and 7 kD fractions. The two fractions which differentially stimulated larval and pupal prothoracic glands *in vitro* have been termed 'big' and 'small' PTTH respectively. Both forms were demonstrated to be peptides by the use of

proteolytic enzymes which decreased the *in vitro* activity by 95%. The composition and structure of the two forms have yet to be determined.

Agui *et al.* (1979) demonstrated that PTTH was produced by a single neurosecretory cell in each hemisphere of the protocerebrum of the *Manduca* brain. These same workers in 1980 further demonstrated that the corpus allatum contained far greater PTTH activity than the corpus cardiacum; activity which corresponded with PTTH activity in the brain over the same developmental period; and concluded that in *Manduca*, the corpus allatum is the neurohaemal organ for PTTH. The pair of contralateral cells innervating the corpus allatum of *S. crassipalpis* (Giebultowicz and Denlinger, 1986) have somata located in the same region of the brain as the *Manduca* group III cells (Buys and Gibbs, 1981) and may therefore be a likely source of PTTH. The organisation of the brain/retrocerebral complexes in *Manduca* and *Sarcophaga* are quite different however, and assuming that the cells are homologous, the neurosecretory axons in *Sarcophaga* must transverse the region of the prothoracic glands en route to the site of PTTH release in the corpus allatum.

In the larvae and pupae of higher diptera, the prothoracic glands, the corpus cardiacum and corpus allatum are fused together into a structure known as the ring gland (King *et al.*, 1966), rather than distinct corpora allata and prothoracic glands as found in *Manduca*. The ring gland is located on the dorsal surface of the brain attached at the corpus cardiacum (See figure 4.1). The side arms comprise the prothoracic glands and where they rejoin, the corpus allatum. The aorta passes directly through the centre and must be cut to allow the removal of the gland which is also associated with a pair of trachea that are very useful for handling purposes. In this study, no attempt was made to separate the prothoracic glands from the corpus allatum and

corpus cardiacum, instead the whole ring gland was used for *in vitro* incubation studies.

The role of the prothoracic glands has been shown with few exceptions to be one of producing and secreting a factor previously known as the moulting hormone (Gilbert and King, 1973). Of the polyhydroxylated ketosteroids with moulting hormone activity, 20-hydroxyecdysone was observed to be more active than ecdysone. Insects were shown to be capable of converting ecdysone to 20-hydroxyecdysone *in vivo* by King and Siddall (1969) but the prothoracic glands themselves were not able to effect this conversion (King, 1972). The development of an *in vitro* culturing technique for *Manduca* prothoracic glands (Vedeckis and Gilbert, 1973) and a radioimmunoassay for ecdysteroids (Borst and O'Connor, 1972) allowed the conclusion that ecdysteroids were indeed secreted by the prothoracic glands (Gilbert *et al.*, 1980). Culture medium in which prothoracic glands had been incubated yielded RIA positive material with ecdysteroid activity. In contrast, no activity was noted in extracts of the glands indicating that this material was released immediately following synthesis. The RIA specificity indicated that the ecdysteroid produced was ecdysone. This supported work by King *et al.* (1974) who presented a complete chemical analysis involving high performance liquid chromatography, RIA, bioassay, and mass spectroscopy. A similar study of *in vitro* synthesis by *S. bullata* ring glands (Bollenbacher *et al.*, 1976) also established that ecdysone rather than any other ecdysteroid was synthesized. Although Redfern (1984) found that *Drosophila* ring glands sometimes synthesized 20-deoxymakisterone A under certain dietary conditions, it is assumed that the major steroid produced by the prothoracic glands is ecdysone. This forms the prohormone to the more active 20-hydroxyecdysone, the hydroxylation taking place in other tissues, notably

the fat body (Bollenbacher *et al.*,1977).

The role of PTTH to act upon the prothoracic glands to stimulate ecdysone synthesis and the subsequent moulting process has long been one of the basic tenets of insect endocrinology (Gilbert and King,1973, Bollenbacher *et al.*,1977). Williams (1947,1952), using the diapausing cecropia silkworm *Hyalphora cecropia*, demonstrated conclusively that the prothoracic gland was the target of the brain's tropic action. To initiate development, an activated brain required the presence of an active prothoracic gland. Truman and Riddiford (1974) and Bollenbacher *et al.* (1975) noted two peaks of prothoracic gland activity in the last larval instar of *Manduca* which coincided with two proposed periods of PTTH release. The haemolymph ecdysteroid titre followed these proposed periods of PTTH release, thereby providing more evidence for PTTH acting on the prothoracic glands.

Gilbert *et al.* (1980) proposed that PTTH, in common with vertebrate steroidogenic peptides such as adrenocorticotrophic hormone (ACTH) and luteinizing hormone (LH), exerts its effect on the prothoracic glands though the action of a cyclic adenosine monophosphate (cAMP) secondary messenger. This cAMP involvement was first suggested by the ability of phosphodiesterase inhibitors to stimulate ecdysone synthesis by the prothoracic glands of *Manduca* (Vedeckis *et al.*,1976), and to mimic the neurohormonal-mediated changes in membrane potential in the prothoracic glands of the wax moth *Galleria mellonella* (Gersch and Birkenbeil,1980). Smith *et al.* (1984) demonstrated that PTTH action in *Manduca* was mimicked by agents that act by increasing intracellular levels of cAMP such as 1-methyl-3-isobutylxanthine (MIX), a potent phosphodiesterase inhibitor, cAMP analogues, and forskolin, a diterpine which increases adenylate cyclase activity

(Seamon *et al.*,1981). Smith *et al.* (1984) further demonstrated that partially purified big PTTH stimulated the formation of cAMP in both day 3 fifth instar larval and day 0 pupal prothoracic glands, but that a significant accumulation only occurred in the larval glands. The pupal glands only showed this accumulation in the presence of a phosphodiesterase inhibitor. These workers proposed that cAMP was involved as the secondary messenger to PTTH, but that the developmental status of the prothoracic glands influenced the degree to which cAMP accumulated. It was suggested that the accumulation of cAMP in larval glands is required to overcome a lower level of cAMP-dependent protein kinase, the next step in the chain towards ecdysone synthesis.

Similar responses to those discussed above were noted by Shapiro (1983) in the mosquito ovary. In mosquitoes, ecdysone is produced by the adult ovary as a regulatory agent for vitellogenesis, its synthesis being controlled by the neuropeptide egg development neurosecretory hormone (EDNH) (Hagedorn,1984). Exposure of ovaries to EDNH *in vitro* results in a rapid dose dependent accumulation of cAMP which could be increased by the action of MIX. This evidence was used to suggest that neuropeptide regulation of steroidogenesis in insects may be controlled by similar intracellular mechanisms regardless of the target tissues involved (Smith *et al.*,1984).

A subsequent study by Smith *et al.*(1985) demonstrated that the steroidogenic effects of 'big' PTTH are dependent upon extracellular calcium. When calcium ions are removed from the incubating medium, no response to PTTH was noted from *Manduca* prothoracic glands. The use of the calcium ionophore A23187, an antibiotic which increases the permeability of biological membranes to divalent cations (Pfeiffer *et al.*,1978), stimulated ecdysone synthesis in the absence of PTTH. Both PTTH and A23187 enhanced the

formation of cAMP, as measured by the intracellular conversion of [ $^3\text{H}$ ]ATP to [ $^3\text{H}$ ]cAMP; a conversion which was found to be calcium dependent. By contrast, the steroidogenic effects of dibutyryl cAMP, MIX and forskolin were not dependent upon extracellular calcium. On the basis of these two studies (Smith *et al.*, 1984,1985), these workers proposed a model for the involvement of calcium and cAMP in PTTH stimulated ecdysone synthesis (Smith *et al.*,1985; Smith and Gilbert,1986). They suggested that PTTH binds to receptors, located on the plasma membrane of the prothoracic gland cells, which are associated with a hormone-sensitive calcium channel and/or a membrane bound calcium sensitive adenylate cyclase system. The subsequent influx of calcium ions into the cells through the calcium channels opened by the binding of PTTH is proposed to stimulate the conversion of ATP to cAMP by the adenylate cyclase. The cAMP then activates a cAMP-dependent protein kinase responsible for phosphorylating and thereby enhancing the activity of a rate limiting enzyme(s) involved in the synthesis of ecdysone. The existence of a dual function for PTTH in cAMP synthesis is supported by similar observations of other single hormone or neurotransmitter systems in which both cAMP and calcium are involved (Review in Rasmussen,1981). Smith and Gilbert (1986) further proposed the involvement of calmodulin, a protein responsible for mediating the effects of calcium on cAMP formation. A stimulatory effect of calmodulin on adenylate cyclase is well documented in vertebrate nervous tissues (Bronstrom *et al.*,1978) and calcium ion/calmodulin-sensitive adenylate cyclases have recently been described for insect tissues (Combest *et al.*,1985). A stimulatory effect of calcium/calmodulin on phosphodiesterase activity has been observed in several systems (Cheung,1980). Preferential stimulation of this enzyme in pupal prothoracic glands from *Manduca* may help to explain the absence of accumulation of cAMP at this stage of development. A

comprehensive review of the involvement of cyclic nucleotides in hormone action in insects is given in Smith and Combest (1985).

Diapause in many species of insects results from a failure of the brain/prothoracic gland to initiate development by the synthesis and release of ecdysone (review in Denlinger, 1985). Williams (1947, 1952) demonstrated that the prothoracic gland was essential for the initiation of development from diapause in the cecropia silkworm *Hyalophora cecropia*. Meola and Adkisson (1977) confirmed the necessity of the prothoracic gland for initiating adult development in the pupae of the bollworm *Heliothis zea*. When prothoracic glands were removed from young diapausing pupae, none developed on transfer to higher temperatures. However, if a prothoracic gland was implanted, or injections of ecdysone given, development promptly ensued, suggesting that an active prothoracic gland or its product is essential for diapause termination.

Bowen *et al.* (1984a) suggested that the virtual absence of ecdysteroids in diapausing *Manduca sexta* was a consequence of the failure of the diapausing pupal prothoracic glands to synthesize the steroid hormone. They proposed that this was due to the curtailment of PTTH release, as opposed to a failure to synthesize or transport the hormone, and to the development of refractoriness of the prothoracic glands to PTTH stimulation. They demonstrated that diapausing brains contained at least as much PTTH as non-diapausing brains, as measured by *in vitro* activation analyses of isolated prothoracic glands. Glands from developing pupae maintained their ability to respond to a standard dose of PTTH by synthesizing ecdysone, whereas those from diapausing pupae gradually lost the ability and became refractory. However, a degree of residual basal synthesis of ecdysone persisted into



diapause at a level which could not be increased by PTTH activation. Smith *et al.* (1985) further demonstrated that this basal synthesis could not be increased by the action of dibutyryl cAMP, A23187 or MIX. These workers therefore showed that in diapausing *Manduca* prothoracic glands, the cellular basis of refractoriness occurred beyond the level of cAMP formation, possibly at the level of the cAMP dependent protein kinase. Diapausing prothoracic glands actually synthesized more cAMP in response to PTTH activation, but no ecdysone was produced. They suggested that this accumulation may result from an alteration in the regulatory and/or catalytic unit of the adenylate cyclase system.

In flesh flies, juvenile hormone (JH) may have some importance prior to and during diapause as well as at the onset of adult development (Walker and Denlinger, 1980; Denlinger, 1981a, 1981b). In non-diapausing pupae, a single pulse of JH precedes the rise in ecdysone associated with adult development. In diapause destined individuals, JH activity is high at pupariation and pulses continue at 24h intervals throughout pupation and the onset of diapause. These pulses may correspond with daily cycles of metabolic activity, as measured by oxygen consumption. Upon diapause termination, a pulse of JH activity appears within 3h (Walker and Denlinger, 1980). This pulse may be equivalent to that noted in non-diapause destined pupae prior to adult development. These workers suggest that JH may have an indirect role in promoting the action of 20-hydroxyecdysone by priming the tissues to respond.

Diapause termination in flesh flies is not effected by photoperiod (Denlinger, 1972; Ohtaki and Takahashi, 1972), presumably because most pupae remain buried 4-8cm underground where photoperiodic cues can not be

detected (Unpublished observations cited in Denlinger,1981a). Temperature however, does play an important role in the initiation of adult development. Although chilling is not a prerequisite for adult development in *Sarcophaga*, as it is in *H. cecropia* (Williams,1956), it can significantly shorten diapause upon return to higher temperatures (Fraenkel and Hsiao,1968a; Ohtaki and Takahashi,1972). The mechanisms of this temperature response are as yet unknown but appear to be linked with the resumption of endocrine activity.

Fraenkel and Hsiao (1968b) demonstrated that diapause could be broken by the injection of ecdysone into the pupae of *Sarcophaga argyrostoma*. Zdarek and Denlinger (1975) showed that the duration of subsequent post-injection pupal diapause in *S. crassipalpis* was dependent on the dose of ecdysone injected, higher doses greatly shortening the duration. The pupal diapause of the Bertha armyworm *Mamestra configurata* (Bodnaryk,1977) was shown to be a dynamic rather than a static state by the requirement for different effective doses of 20-hydroxyecdysone to initiate adult development from pupae of different ages. It is suggested that ecdysone or 20-hydroxyecdysone may exert a stimulatory action on the brain or prothoracic gland through a positive feedback mechanism, thereby effecting the resumption of endocrine activity (Denlinger,1985).

Evidence is growing for the proposal that diapause termination is not a brain/prothoracic gland centered event. Smith *et al.* (1986) proposed that termination may be dependent upon the release of tropic factors, possibly PTTH, that ultimately stimulate steroidogenesis. However, the observations by Denlinger and Wingard (1978), that cGMP can break diapause when injected into *S. crassipalpis* pupae, but that cAMP can not, indicates a recovery initiation mechanism outside the realm of PTTH action. Bodnaryk (1987)

demonstrated that the injection of dibutyryl-cAMP into diapausing bertha armyworm (*Mamestra configurata*) pupae actually reduced diapause termination to zero, whereas cGMP increased termination to more than 90%, both compared to a control level of 15%. He proposed that cAMP acts to maintain diapause in this species. Gray *et al.* (1986) demonstrated that a humoral factor, present in the fat body of diapausing *Heliothis zea* pupae, was required along with PTTH for diapause recovery. This factor was absent from the haemolymph whilst the pupae were maintained at diapause sustaining temperatures. Whilst *H. zea* is unusual in that diapausing pupae release PTTH for adult development at the onset rather than at the termination of diapause (Meola and Adkisson, 1977), the release of a factor not related to PTTH required for the initiation of adult development may be more common than presently thought. Watson *et al.* (1985) demonstrated the existence of a haemolymph prothoracicotropic factor (HPF) which was able to stimulate ecdysone synthesis in both larval and pupal *Manduca* prothoracic glands without enhancing cAMP formation. This factor is apparently identical to an agent produced by the *Manduca* fat body in response to JH stimulation (Gruetzmacher *et al.*, 1984a, 1984b). The mechanisms of diapause termination, whilst not fully understood, will be considered more fully in the discussion.

Throughout this introduction, the technique of *in vitro* prothoracic gland activation has been referred to. This technique which was first developed for isolated *Manduca* prothoracic glands by Bollenbacher *et al.* (1979) consists of incubating a gland in a small volume (25 µl) of medium at an appropriate temperature for an appropriate period. PTTH extracted from *Manduca* brains was added to this medium and a classic sigmoidal dose response curve obtained. The actual determinations of activation were made by ecdysteroid RIA on aliquots of the incubation media after the removal of the glands.

*Manduca* prothoracic glands consist of two contralateral clusters of cells which can be removed separately. One gland can be subjected to an activation treatment whilst the other can be used as a control thereby allowing the most accurate elimination of differences between individuals. Roberts *et al.* (1984) modified this technique for use with ring glands from *S. bullata*. They obtained similar dose response curves using groups of ring glands for each treatment rather than the paired prothoracic glands used by Bollenbacher *et al.* (1979). This technique has been further modified for use in the course of the present investigation.

This chapter will concentrate on the cellular basis of PTTH action on isolated ring glands from *Sarcophaga argyrostoma* and *Calliphora vicina*. The effect of PTTH extracted from *Sarcophaga* brains on ring glands from both species will be determined, as will the effects of agents reported to raise the intracellular levels of cAMP and cGMP. As the two species enter diapause, one as a pupa (*Sarcophaga*) and the other as a larva (*Calliphora*), the ability of the ring glands to respond to PTTH stimulation will be followed. The role of cAMP in diapause will also be investigated. Upon a transfer from a lower temperature, at which diapause is maintained, to a higher temperature calculated to break diapause, the changes in ring gland competency will be followed and the role of the brain/ring gland complex in this onset of adult development will be examined.

## Materials and Methods

### Culture maintenance

Stock cultures of 'FAST' strain *Sarcophaga* (See chapter 1) and *Calliphora* were raised in cages at 25°C under continuous light as described in the general methods section. Diapause destined *Sarcophaga* were raised by maintaining the adults at 25°C, and then transferring the larvae to 18°C, both stages under LD 12:12. This temperature reduction ensured the near saturated pupal diapause incidence deemed necessary for this portion of the investigation. Diapause larvae of *Calliphora* were produced by keeping the adults at 20°C and LD12:12. Eggs deposited on day 12 were then raised at 11°C in constant darkness (Saunders *et al.*, 1986). This ensured a high larval diapause incidence. To break diapause in both species, the cultures were transferred to 25°C under constant darkness.

### Ecdysteroid radioimmunoassay

Radioimmunoassay (RIA) termination was accomplished by donkey anti-rabbit IgG second antibody (Scottish Antibody Production Unit) against Horn-22 antisera (Kindly supplied by Professor L.I. Gilbert, University of North Carolina, Chapel Hill, N.C., USA) against an ecdysone standard curve as described in the RIA methodology appendix. All results are expressed as ecdysone equivalents.

None of the chemicals used in these investigations for ring gland incubation were found to interfere with the RIA.

### In vitro assay

Insects to be used for the *in vitro* assay were anaesthetised on ice for approximately 20 minutes. The posterior half of the larva was cut off and discarded. The anterior half was pinned through the mouth hooks with the dorsal surface uppermost. The preparation was covered with ice cold saline (0.15M) and the dorsal surface slit from the midregion up to the mouth hooks to expose the brain/reterocerebral complex. Ring glands from postfeeding larvae were excised by cutting aortal and tracheal connections and dissecting them free of the surrounding tissues with care not to damage or touch the ring glands. The trachea provided natural devices for the handling of the larval ring glands (see figure 4.1). When whole brain-ring gland complexes were removed, a grip was taken on the nerves exiting the ventral ganglion, and the complexes carefully dissected clear of the surrounding tissues. These dissection methods were used for both *Sarcophaga* and *Calliphora* larvae. To dissect *Sarcophaga* ring glands from individuals up to 24h following pupariation, the cuticle of the anterior three or four segments were removed with a razor blade to expose the brain complex. The removal of ring glands from older and diapausing individuals was accomplished by squeezing the puparium between the fingers to crack open the anterior segment and cutting back the next three or four segment to expose the pupa. The pupa was pinned through the rear of the thorax region and the preparation covered with ice cold saline. An incision in the pupal cuticle was made from the anterior point to behind the head region. The large quantities of dissociated fat cells were removed by flushing the exposed area with saline with a Pasteur pipette. In the first two days of pupal development at 25°C, the ring gland continued to be associated with the dorsal surface of the brain, until the pupa entered diapause. The gland moved in a posterior direction, and became associated with the gut, a section of which could be used as a handle to ease the

manipulation of the gland. Within 24h of diapause termination and the onset of pharate adult development, the ring gland could not be located within the pupa.

In all cases, the excised ring glands and brain-ring gland complexes were transferred to a 10 $\mu$ l hanging drop suspended over Grace's medium (Gibco) in an upturned petri dish to avoid undue evaporation, according to the method of Roberts *et al.* (1984). All incubation solutions were made up with Grace's medium containing an additional 10mM CaCl<sub>2</sub>, with the exception of the EDTA experiment when this was omitted. The hanging drops containing the ring glands were incubated at 25° in darkness. After 4h, the ring glands were removed from the incubation mixtures and two 5 $\mu$ l aliquots assayed directly for ecdysone by RIA.

To prepare the PTTH extract for the *in vitro* assay, brains, with the ring glands removed, were excised from 3 to 4h *Sarcophaga* prepupae (Fraenkel and Bhaskaran, 1973), rinsed and homogenised in Grace's medium, boiled for two minutes to denature and precipitate any proteases, and centrifuged at 13000 x g for 5 minutes. The resulting supernatant was designated PTTH extract and was used for both the *Sarcophaga* and *Calliphora* incubation studies. Activation of the ring glands by the PTTH extract was demonstrated using a dose response protocol (Bollenbacher *et al.*, 1979). Activation was expressed as the amount of ecdysone produced by the experimental gland or as the activation ratio, the amount of ecdysone produced by the experimental ring gland (+PTTH) divided by the amount produced by the control (-PTTH) during the same period. Extracts of ventral ganglia were prepared as controls.

Other extracts were prepared as described in the relevant sections. Cyclic nucleotides, EDTA, and MIX, (1-methyl-3-isobutylxanthine) were purchased

from the SIGMA Chemical Co. The concentrations of reagents used in these investigations were based on those used by Smith *et al.* (1984,1985).



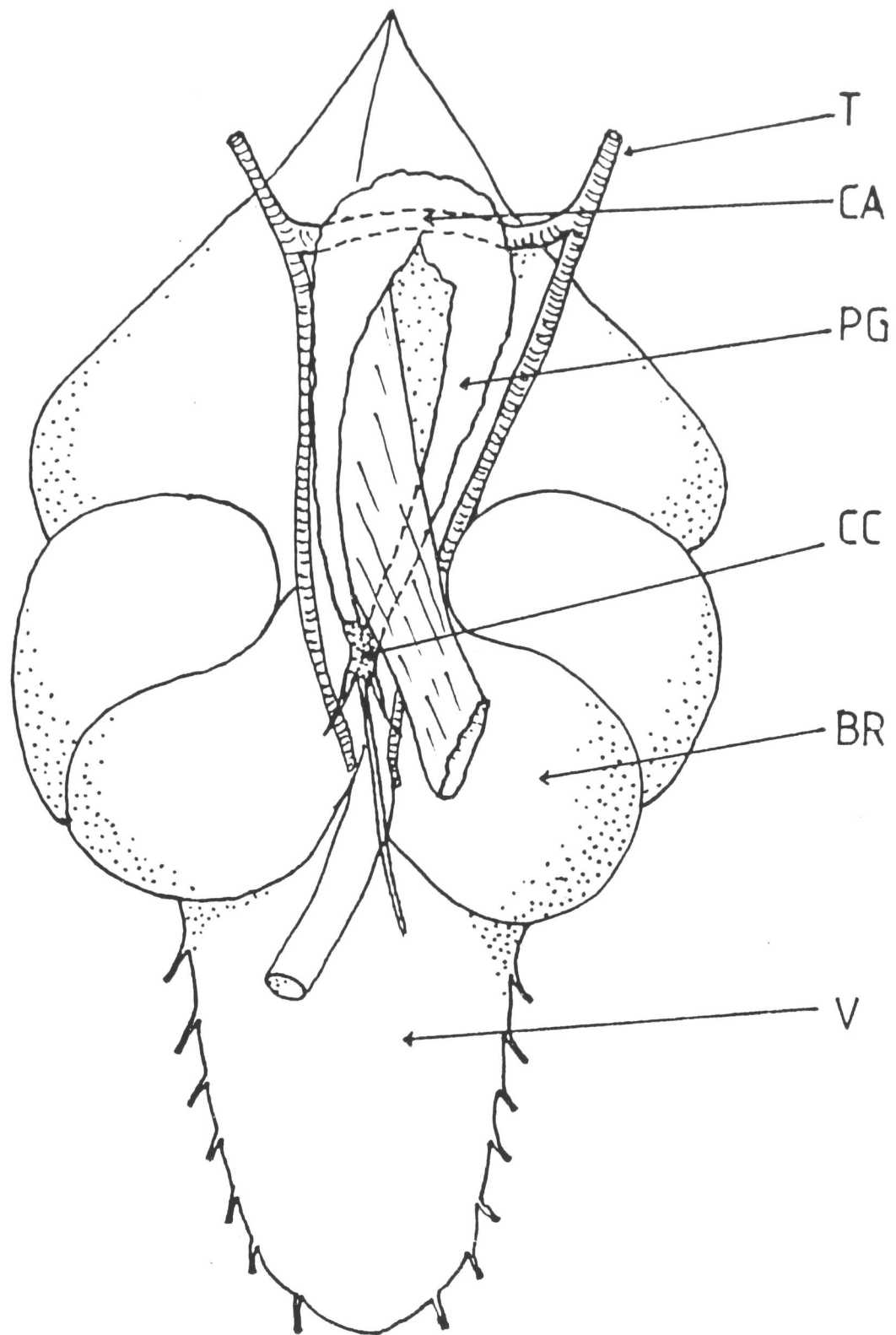


Figure 4.1: The anatomy of the brain/ring gland complex from *Sarcophaga* spp. showing the position of the corpus allatum (CA), corpus cardiacum (CC) and the prothoracic gland (PG) which together comprise the ring gland; and the brain (Br), ventral nerve mass (V) and trachea (T) attached to the ring gland. After Giebultowicz and Denlinger (1986).

## Results

### 1. In vitro activation of the ring gland by prothoracicotropic hormone

The activation of isolated *Sarcophaga* post feeding larval ring glands by prepupal (Fraenkel and Bhaskaran, 1973) brain extracts, assumed to contain prothoracicotropic hormone (PTTH), is shown in figure 4.2. This work followed the protocol proposed by Roberts *et al.* (1984). The glands did not respond to PTTH stimulation until more than 0.25 brain equivalents were administered. At this concentration, the amount of ecdysone produced increased from the basal level of less than 500 pg/4h to over 1300 pg/4h, an activation ratio approaching 3. No increase in ecdysone production was noted when the glands were incubated with a control extract from one ventral ganglion. Ring glands removed from red spiracular larvae of *Sarcophaga* were more active and more responsive to PTTH stimulation than those from post-feeding larvae. A marginally higher basal level of ecdysone synthesis ( $620 \pm 80$  pg/4h) increased steadily with increasing PTTH concentration to a maximum of over 2100 pg/4h. Ecdysone production maxima were reached in both dose response curves when the PTTH concentration reached 0.5 brain equivalents.

### 2. The effects of cyclic nucleotides on isolated ring glands in vitro

Following the dose response experiments, a 0.5 brain equivalent measure of 4h prepupal *Sarcophaga* brain extract was adopted as the standard PTTH dose. In each experiment, a blank was included which consisted of a group of ring glands ( $n=5$ ) from the appropriate developmental stage, incubated in Grace's medium to determine the basal level of ecdysone production. Figure 4.3 shows that the inclusion of 0.1mM 1-methyl-3-isobutylxanthine (MIX), a

potent phosphodiesterase inhibitor which increases intracellular cyclic nucleotide levels (Ravankar and Robins, 1982), in the incubation medium increased the level of ecdysone production from both the basal and PTTH stimulated levels in *Sarcophaga*. The removal of calcium ions by the inclusion of 10mM EDTA negated the effect of PTTH thereby lowering the amount of ecdysone produced to basal levels.

The involvement of cyclic nucleotides in the mediation of PTTH action in *Sarcophaga* is more fully investigated in figure 4.4. The cyclic adenosine monophosphate (cAMP) analogues dibutyryl-cAMP and 8-bromo cAMP (10mM) were investigated for their effects on ecdysone production. Dibutyryl-cAMP which enters cells more rapidly than cAMP (Ravankar and Robins, 1982) was associated with a very large increase in ecdysone synthesis, whereas 8-bromo cAMP which is more resistant to degradation than cAMP was not. The cyclic guanosine monophosphate (cGMP) analogues, dibutyryl cGMP and 8-bromo cGMP had little effect on the basal levels of ecdysone production.

Figure 4.5 shows data obtained from *Calliphora* ring glands exposed to the standard *Sarcophaga* brain extract. The large increase from basal levels to 1500 pg/4h demonstrates a degree of similarity in response to PTTH and possibly therefore in PTTH structure and function. Similar increases in ecdysone production to those in *Sarcophaga* were associated with cyclic nucleotide stimulation.

### **3. The competency of ring gland function in diapause**

The cross hatched bars of figures 4.4 and 4.5 refer to ring glands removed from day 28 post pupariation diapausing *Sarcophaga* pupae (figure 4.4) and day 32 post oviposition diapausing *Calliphora* larvae (figure 4.5). The basal level of

ecdysone production by *Sarcophaga* is below the limit of detection of the radioimmunoassay used (30 pg). This basal level did not rise even when PTTH was added to the medium. No stimulation of ecdysone production was noted with 10mM Dibutyryl cAMP either, indicating that a block to ring gland function existed further downstream from the cyclic nucleotide link.

Figure 4.5 shows similar data obtained from diapausing *Calliphora* ring glands. Whilst there is no increase in ecdysone production associated with PTTH or Dibutyryl cAMP treatment, the basal levels of synthesis are much higher than those for *Sarcophaga*. The diapause basal level is the same as the non-diapause basal level. (c 300–400 pg/Ring gland/4h).

#### 4. Time course of ring gland deactivation in diapause

Larvae of *Sarcophaga* were raised under diapause inducing conditions and newly formed puparia collected. Groups of ring glands (n=5 to 10) were removed daily and incubated in the standard 0.5 brain equivalent PTTH dose. Figure 4.6 shows the changing responses of the pupal ring glands as the pupae enter diapause. The initial three days show high ecdysone production levels, and therefore a high level of ring gland competency to respond to PTTH (c 2200 pg ecdysone/4h). As the pupae entered diapause between day 3 and day 4 post pupariation, the glands became refractory to PTTH stimulation, and the level of ecdysone production fell to the limit of detection.

When the competency of diapause destined ring glands from *Calliphora* larvae was followed (Figure 4.7), the initial ecdysone production level in post feeding larvae was c 900 pg/4h. This level fell between day 22 and 28 post-oviposition, as the larvae entered diapause, indicating either a more gradual loss of competency than in *Sarcophaga* or a lack of synchrony within

the population, or both. The ring glands continued to produce ecdysone at a basal level of  $\approx 250$  pg/4h with no elevation by PTTH stimulation. These results contrast with those from *Sarcophaga* which showed a very rapid transition into diapause and a complete absence of residual basal competency. Despite this basal synthesis rate, the level of circulating ecdysteroids in the haemolymph of diapausing *Calliphora* is very low (figure 4.9), close to the limit of detection.

#### 5. The recovery of ring gland competency upon diapause termination

14 days after pupariation, a group of diapausing *Sarcophaga* pupae were transferred to 6°C. Sixty-seven days after pupariation, these were then transferred to 25°C, and the competency of the ring glands determined against the standard dose of PTTH *in vitro*. Figure 4.8 shows that the ring glands resume the ability to respond to PTTH within 24h. The levels of ecdysone synthesized are lower than those produced by the pupal ring glands before the onset of diapause ( $\approx 900$ pg/4h), but do indicate a resumption of activity. Ring gland competency could not be followed beyond 24h, after the temperature transfer, because of difficulties in locating and removing glands as pharate adult development commenced. Ring glands from pupae which were maintained in 6°C did not regain competency to respond to PTTH.

Figure 4.9 demonstrates the rapidity with which diapausing *Calliphora* larvae can respond to a temperature increase from 11°C to 25°C in constant darkness. Day 52 larvae also regained normal ring gland competency within 24h and all had pupariated within 36h. There was a degree of spread of pupariation times between 12h and 36h after the temperature change. Those larvae maintained at 11°C, continued to produce basal levels of ecdysone

regardless of PTTH stimulation.

Brain-ring gland complexes from diapausing *Calliphora* larvae did not regain competency to respond to PTTH stimulation following a temperature increase from 11°C to 25°C *in vitro* (Table 4.1). Only when the brain-ring gland complex was *in vivo* for the 24h warming period did the ring gland respond by regaining competency. The ring glands removed from diapausing brain-ring gland complexes incubated at 25°C continued to secrete ecdysone at the basal level. Brain-ring gland complexes from day 12 non-diapausing *Calliphora* larvae could be maintained at 25°C for 24h and the ring glands would still maintain normal competency to respond to PTTH.

#### 6. The levels of PTTH in diapausing *Sarcophaga* brains

It has been established that the prothoracic glands of *Sarcophaga* and *Calliphora* lose their *in vitro* competency to respond to PTTH stimulation when in diapause. The question of whether PTTH synthesis or storage is altered in diapause is addressed in figure 4.10. This dose response curve for post feeding *Sarcophaga* larval ring glands to day 35 post pupariation *Sarcophaga* diapausing brain extracts indicates that similar levels of PTTH are maintained in diapausing brains as in non-diapause destined prepupal brains (figure 4.2). The level of PTTH in 94h post-pupariation diapause destined pupal brains is also comparable with the non-diapause destined level.

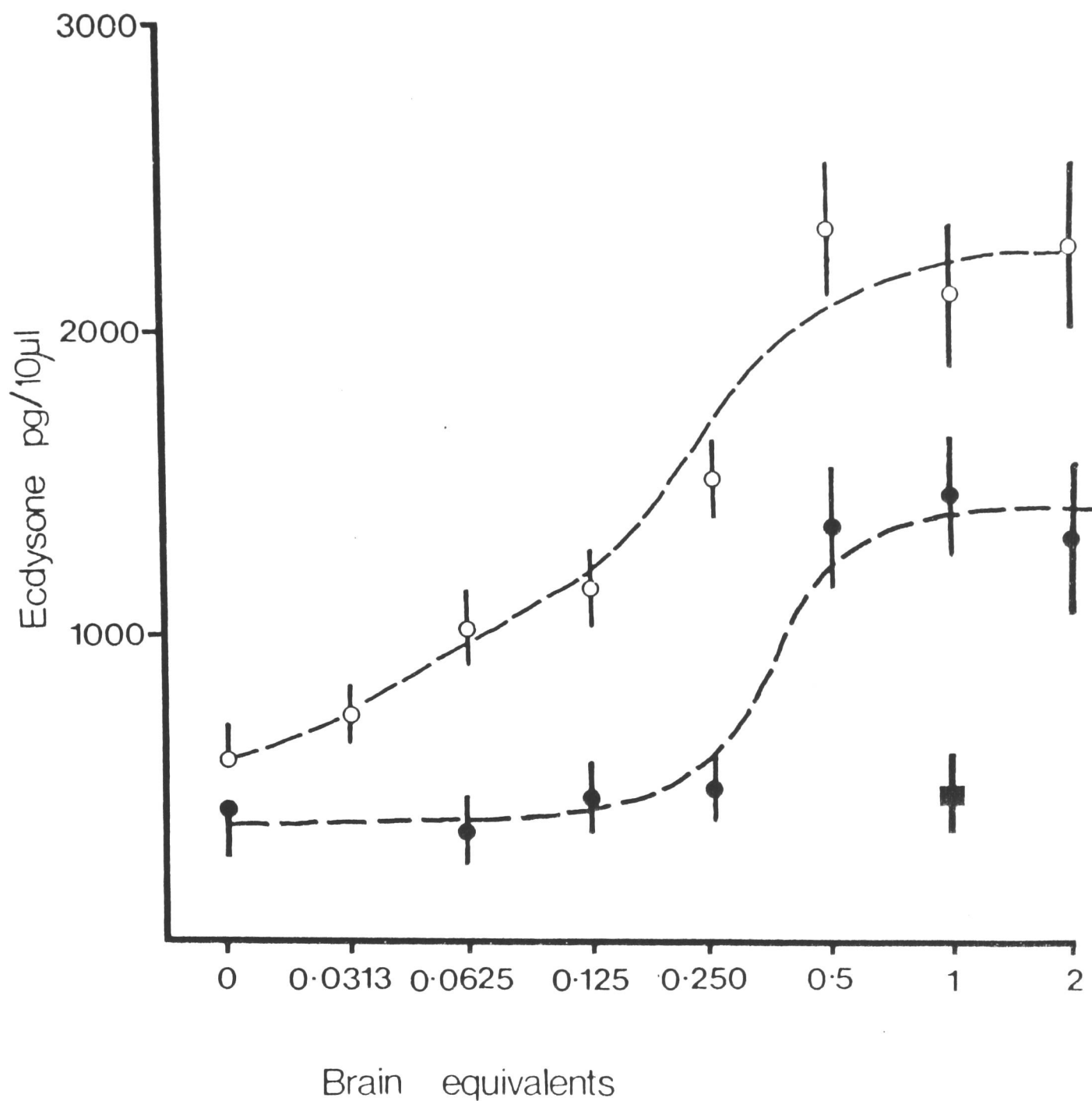


Figure 4.2: Dose response curves for the *in vitro* activation of *Sarcophaga* post-feeding larval (●---●) and red spiracled larval (○---○) ring glands incubated with PTTH extracts from 4h prepupal brains in Grace's medium plus 10mM  $\text{CaCl}_2$  for 4h at 25°C. An extract of ventral ganglion was used as a control (■) with post-feeding larval ring glands. Each point represents the mean  $\pm$  SEM of 5 to 10 separate activation analyses. Results expressed as ecdysone equivalents.

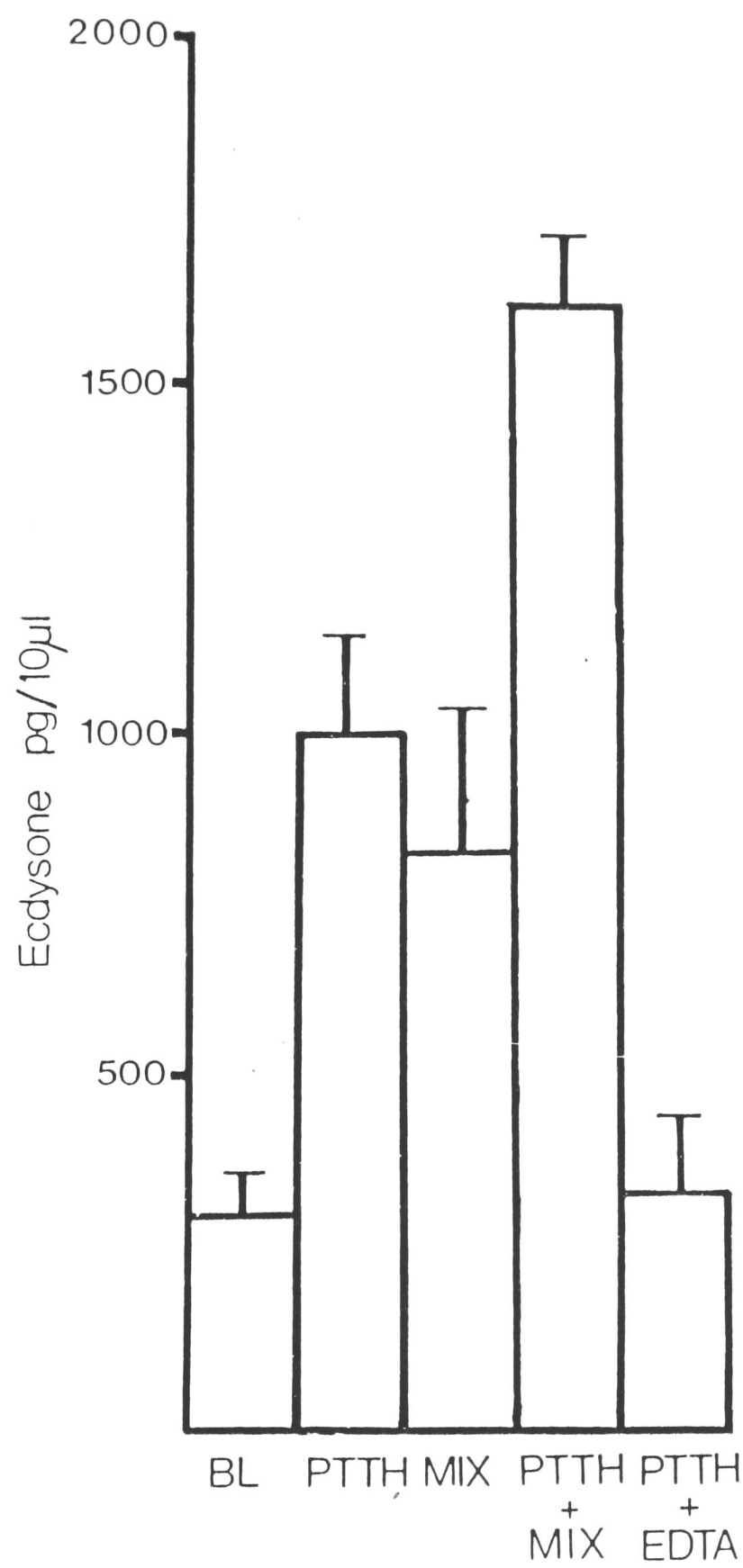


Figure 4.3: The effects of the phosphodiesterase inhibitor, 1-methyl-3-isobutylxanthine (MIX, 0.1mM) and the calcium chelator EDTA (10mM) on the action of PTTH (0.5 brain equivalents) on *Sarcophaga* post-feeding larval ring glands (between gut purge and red spiracle formation) *in vitro*. BL represents the basal level of ecdysone synthesis by these glands. Each point represents the mean  $\pm$  SEM of 5 to 10 separate activation analyses. Results expressed as ecdysone equivalents.



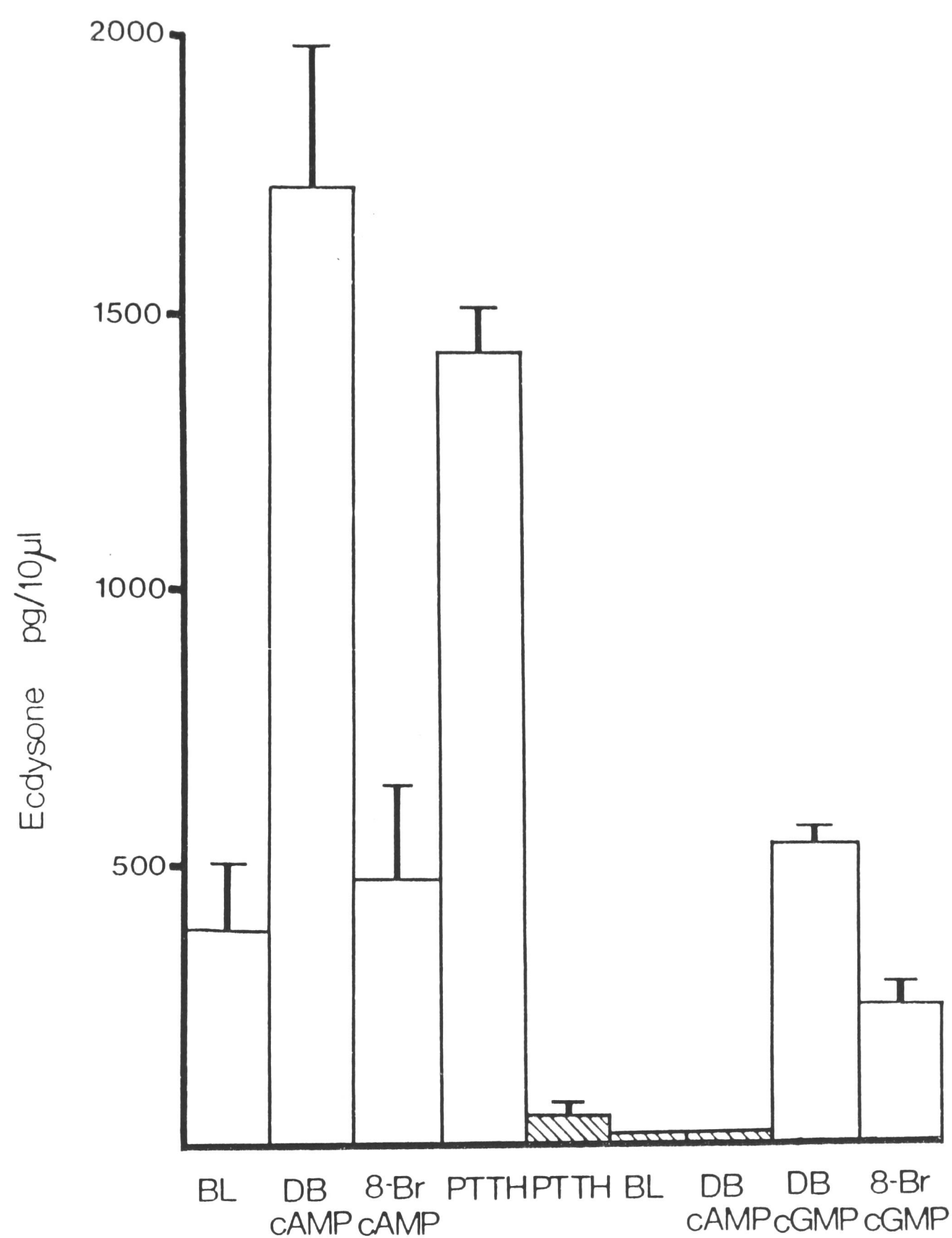


Figure 4.4: The effect of 10mM cyclic nucleotides: dibutyryl cAMP (DBcAMP), 8- bromo cAMP (8-BrcAMP), dibutyryl cGMP (DBcGMP) and 8-bromo cGMP (8-BrcGMP); and PTTH (0.5 brain equivalents) on *Sarcophaga* post-feeding larval ring glands (between gut purge and red spiracle formation) incubated *in vitro* for 4h at 25°C (open bars) and on pupal diapause ring glands (Day 28 post- pupariation) (hatched bars). BL represents the basal levels of ecdysone synthesis by the appropriate ring glands. Each bar represents the mean  $\pm$  SEM of 6 to 10 separate activation analyses. Results expressed as ecdysone equivalents.

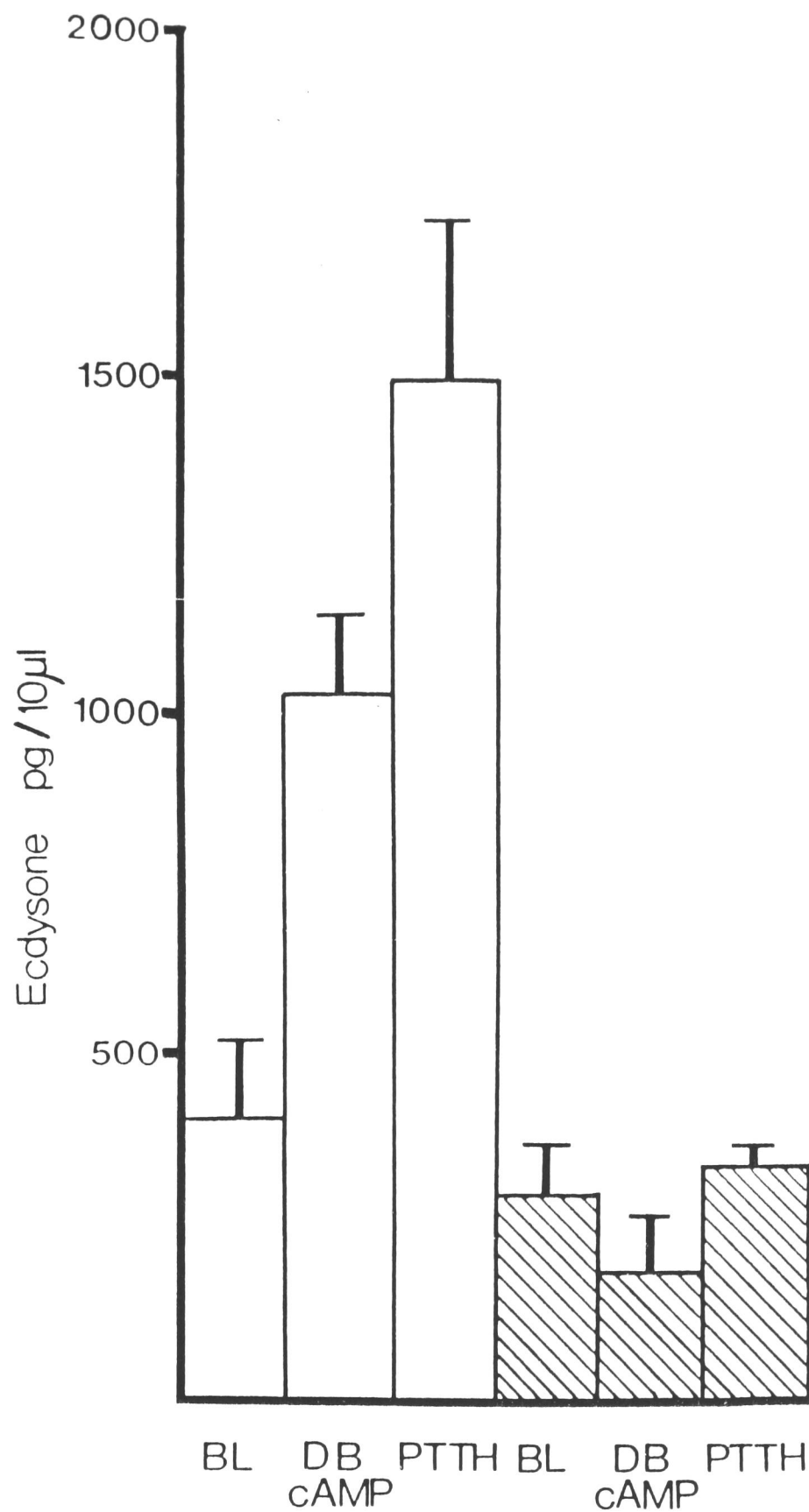


Figure 4.5: The effect of 10mM dibutyryl cAMP (DBcAMP) and PTTH (0.5 brain equivalents from *Sarcophaga* prepupal brains) on ring glands from non-diapause post-feeding (open bars) and Day 32 post-oviposition diapause (hatched bars) *Calliphora* larvae. BL represents the appropriate basal level of ecdysone synthesis. Each bar represents the mean  $\pm$  SEM of 5 to 10 separate activation analyses. Results expressed as ecdysone equivalents.

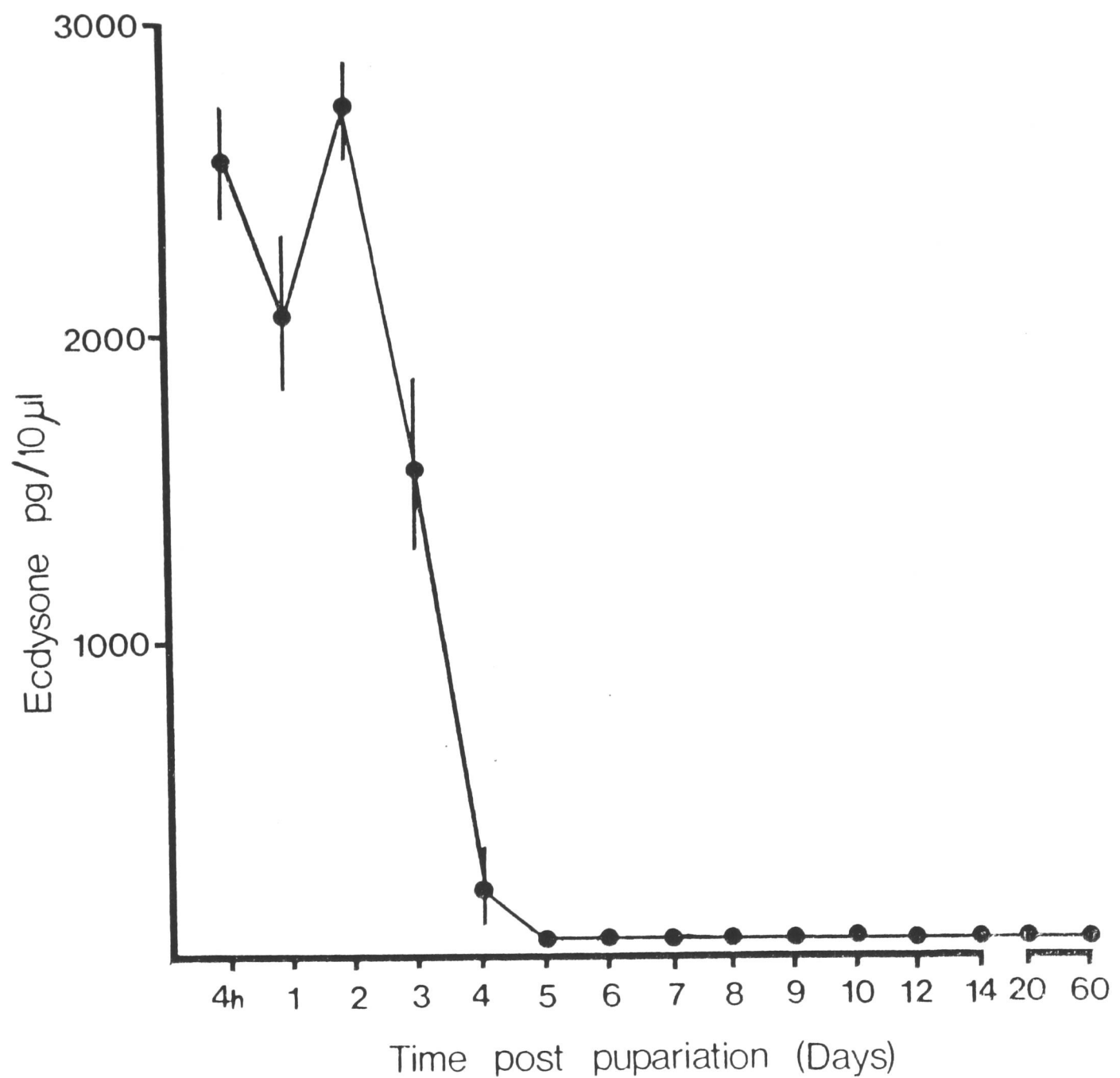


Figure 4.6: The competency of ring glands taken from post-pupariation diapause destined *Sarcophaga* (maintained under LD12:12 at 18°C) to synthesise ecdysone in response to incubation with 0.5 brain equivalents of 4h *Sarcophaga* prepupal brain extract. As the pupae enter diapause (Day 3–4) the ring glands become refractory to PTTH stimulation. Each point represents the mean  $\pm$  SEM of 5 to 10 separate activation analyses. Results expressed as ecdysone equivalents.

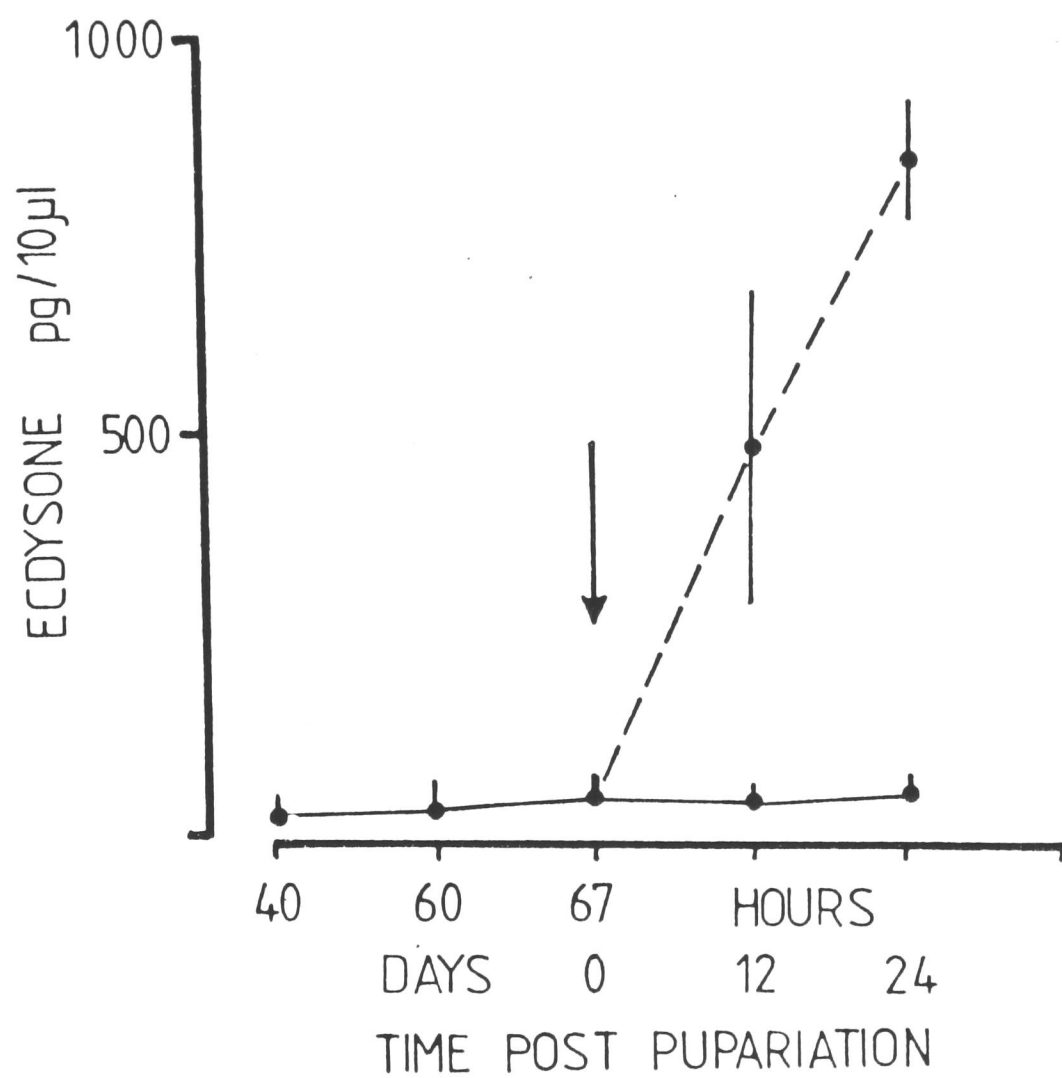


Figure 4.7: The competency of ring glands from diapausing *Sarcophaga* pupae maintained at 6°C to respond to PTTH (0.5 brain equivalents *Sarcophaga* 4h prepupal brain extract) in Grace's medium plus 10mM CaCl<sub>2</sub> (●—●). At day 67 post pupariation (arrow) a group of pupae were transferred to 25°C and tested with PTTH (●---●). Each point represents the mean  $\pm$  SEM of 5-7 separate activation analyses. Results expressed as ecdysone equivalents.

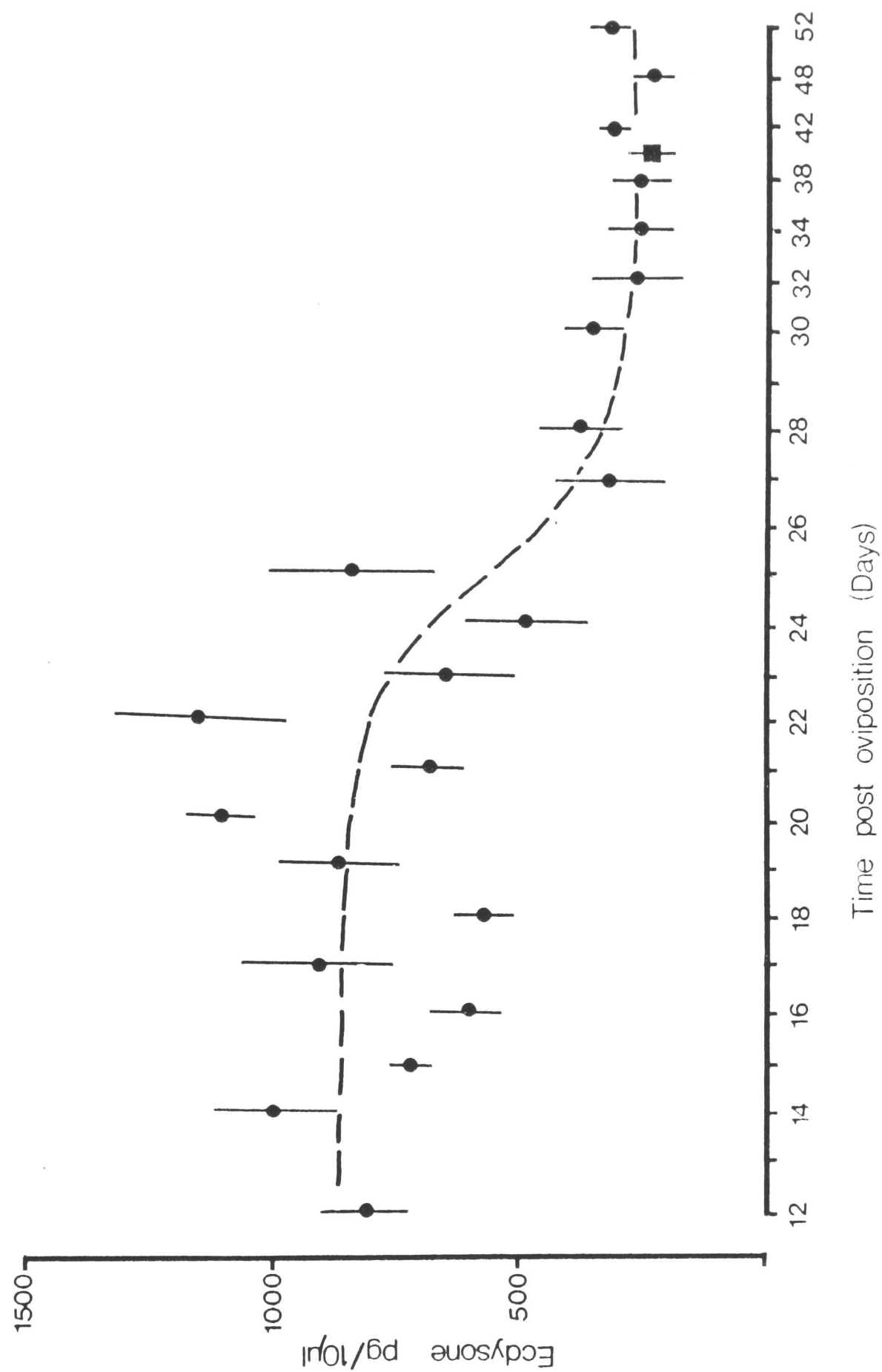


Figure 4.8: The competency of ring glands, taken from diapause destined *Calliphora* larvae (maintained at 11°C in darkness) to synthesise ecdysone in response to incubation with PTTH (0.5 brain equivalents *Sarcophaga* 4h prepupal brain extract) in Grace's medium plus 10mM CaCl<sub>2</sub> as the larvae entered diapause ( --- ). The basal level of ecdysone release by unstimulated ring glands (■). Each point represents the mean  $\pm$  SEM of 5-9 separate activation analyses. Results expressed as ecdysone equivalents.

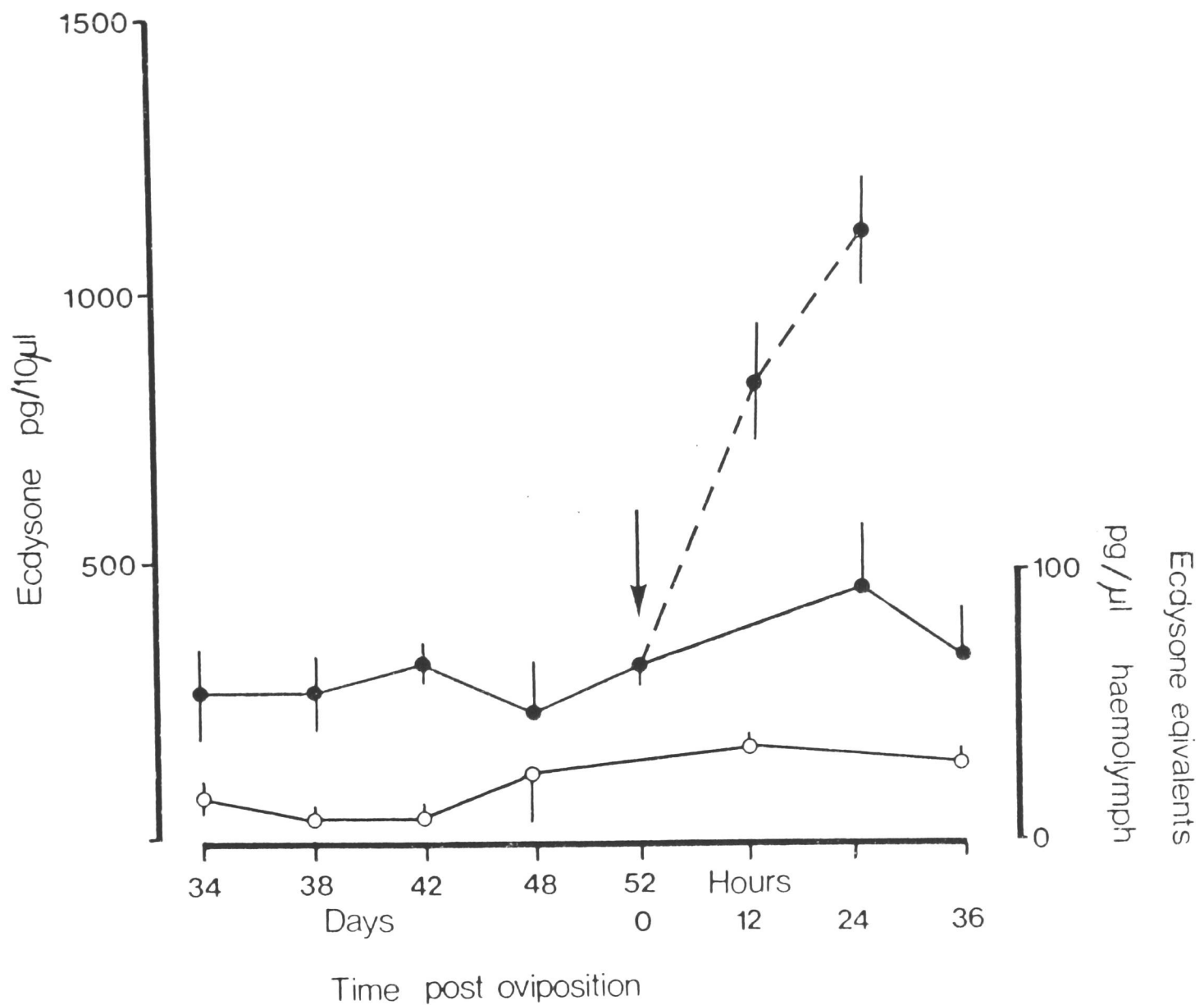


Figure 4.9: The competency of diapausing *Calliphora* larval ring glands to respond to PTTH (0.5 brain equivalents *Sarcophaga* 4h prepupal brain extract) in Grace's medium plus 10mM  $\text{CaCl}_2$  when maintained at 11°C (●—●). At day 52/0h (arrow) a group of larvae were transferred to 25°C and tested with PTTH (●---●). The level of circulating ecdysteroids in the haemolymph at 11°C is shown (○---○). Each point represents the mean  $\pm$  SEM of 5-7 separate activation analyses. Results expressed as ecdysone equivalents.

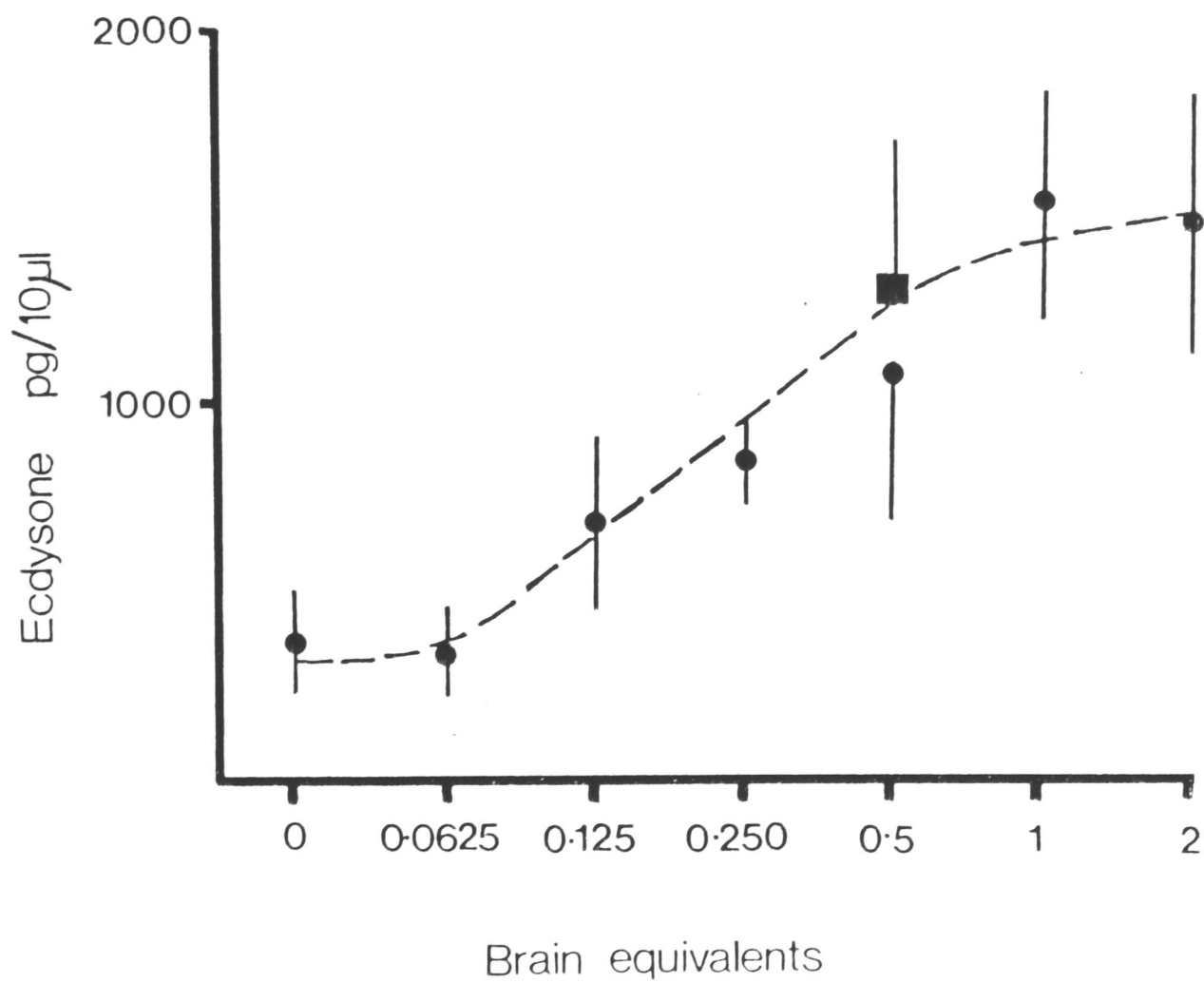


Figure 4.10: Dose response curve for brain extracts from Day 35 post-pupariation diapausing *Sarcophaga* pupae (maintained at 18°C) on post-feeding *Sarcophaga* larval ring glands (●). The closed square (■) shows the effect of a 0.5 brain equivalent dose prepared from a 96h post pupariation (diapause destined) pupal brain. Each point represents the mean  $\pm$  SEM of 5 to 10 separate activation analyses. Results expressed as ecdysone equivalents.

Table 4.1: The effects of a period of warming (24h at 25°C) on the *in vitro* ability of ring glands from diapausing *Calliphora vicina* to respond to 0.5 brain equivalents of *Sarcophaga* prothoracicotropic hormone. Results expressed as pg ecdysone secreted per ring gland per 4h at 25°C. Each figure is the mean ± SEM of 5 to 9 separate activation analyses. Results expressed as ecdysone equivalents.

Treatment	PTTH (0.5 b.e.)	Blank (No PTTH)
Brain-RG warmed	339±120	325±87
Whole animal warmed	1137±100	281±86
Whole animal unwarmed	326±33	315±107



## Discussion

This chapter has focussed on the role of the prothoracic gland in the endocrinology of diapause in *Sarcophaga argyrostoma* and *Calliphora vicina*. Much of the work presented here is based on previous investigations into prothoracic gland function in other species, notably *Sarcophaga bullata* (Roberts *et al.*, 1984) and *Manduca sexta* (Smith *et al.*, 1984, 1985, 1986).

The *in vitro* assay methodology was based on that developed by Bollenbacher *et al.* (1979) for *Manduca sexta*, and Roberts *et al.* (1984) for *Sarcophaga bullata* in which ring glands, containing the prothoracic glands, were incubated in crude brain extracts from 4h non-diapause destined prepupae. No attempt was made to further purify the PTTH beyond the level described by these latter workers. If the proposal of Bollenbacher *et al.* (1984) that two forms of PTTH, 'big' and 'small', is universal, both forms are presumably present in the extracts used in this investigation. The effects of the two forms in the Dipteran species used here is not known, although these workers reported that 'small' PTTH was not as effective at stimulating pupal prothoracic glands from *Manduca* as 'big' PTTH. No significant difference was noted in the activation of *Manduca* larval glands *in vitro*. Roberts *et al.* (1984) noted that ring glands from different developmental stages of larvae and pupae from *Sarcophaga* secreted ecdysone at different rates *in vitro* in the absence of exogenous PTTH stimulation. The glands were presumably synthesizing ecdysone in response to PTTH stimulation prior to their dissection from the animals. The rates corresponded roughly with rises and falls in haemolymph ecdysteroid titres as measured *in vivo*. Glands from

post-feeding larvae synthesized only basal levels of ecdysone. These were chosen for this study as they gave the most reliable indication of prothoracic gland response to stimulation, presumably because they had not recently been stimulated *in vivo*. When ring glands from red spiracled larvae, the stage immediately prior to pupariation, were used, more ecdysone was produced since these glands had already been stimulated by endogenous PTTH. Bollenbacher *et al.* (1983) noted that *Manduca* prothoracic glands achieved half-maximal stimulation after only 30 seconds exposure to PTTH *in vitro*. The glands continued to secrete ecdysone for 2h after removal from the PTTH extract. The higher rate of ecdysone production by red-spiracled larval ring glands *in vitro* may therefore be explained in terms of these observations. The dose response curves obtained in this investigation are similar to that reported by Roberts *et al.* (1984) with *S. bullata* except that the maximum activation they reported was four times higher than that reported here. This may be due to a species difference or to an undetermined difference in experimental protocol. The degree of activation here was more comparable with that reported by Bollenbacher *et al.* (1979) for *Manduca sexta* when an activation ratio (amount produced by activated glands/amount released by unactivated glands) of 4 was reported. Roberts *et al.* (1984) reported an activation ratio of approximately 11.

To investigate the involvement of a cyclic nucleotide secondary messenger system in the action of PTTH, as suggested by Vedeckis *et al.* (1976), isolated ring glands were incubated with agents reported to raise the intracellular levels of cyclic adenosine monophosphate (cAMP) (Smith *et al.*, 1984). Analogues of cAMP were used in this investigation, rather than cAMP itself, since these were either more resistant to degradation, or able to enter cells more easily than the naturally occurring compound (Ravankar and Robins, 1982).

The effects of specific concentrations of these analogues were similar to those reported by Smith *et al.* (1984) in *Manduca sexta*. They reported that an increase in the intracellular concentration of cAMP was associated with and could mimic the steroidogenic action of PTTH. That dibutyryl cAMP stimulated a greater response than 8-bromo cAMP in *Sarcophaga* agrees with the situation reported in *Manduca* pupal prothoracic glands *in vitro*. However, these workers reported that there was little difference in activation by the two nucleotides in larval prothoracic glands. By reducing the catalysis of cAMP in the prothoracic gland cells, the phosphodiesterase inhibitor MIX can bring about an accumulation of intracellular cAMP. This was associated with the increased synthesis of ecdysone noted in the present study. That the PTTH saturated level of ecdysone synthesis could be increased by the action of MIX implies that despite the receptors being saturated, the glands themselves were not maximally producing the steroid hormone. The response of basally synthesizing ring glands to MIX in the absence of exogenous PTTH stimulation suggests that cAMP is produced during basal synthesis of ecdysone as well as being produced during stimulated synthesis.

No steroidogenic effect was noted with the cyclic guanosine monophosphate analogues dibutyryl cGMP and 8-bromo cGMP; the *Sarcophaga* post-feeding larval ring glands continued to secrete ecdysone at the basal level. This agrees with the findings of Smith *et al.* (1984) who noted no activation by cGMP analogues in *Manduca*. At higher concentrations, dibutyryl cGMP actually reduced ecdysone production in this species by 25%. Denlinger and Wingard (1978) reported that cGMP and its derivatives injected into *S. crassipalpis* pupae promptly effected recovery from diapause whereas cAMP had no such effect. Indeed, the injection of cAMP antagonised the effect of co-injected ecdysone which on its own, or with cGMP would break

diapause. Bodnaryk (1987) suggested that cAMP is actually involved in the maintenance of diapause in *Mamestra configurata* whereas cGMP can promptly terminate it. The involvement of cGMP in diapause termination is clear but the observations that cGMP has no stimulatory effect on isolated *M. sexta* prothoracic glands or *S. argyrostoma* ring glands suggest that diapause recovery may be initiated outside the prothoracic gland complex. This will be discussed more fully later in this section.

The involvement of extracellular calcium in PTTH action was indicated in *Sarcophaga* by the addition of the chelating agent EDTA to the PTTH activation medium. Upon subsequent incubation of the ring glands, there was no increase from the basal level of ecdysone secretion. Smith *et al.* (1985) reported a similar effect in *Manduca* where extracellular calcium was required for PTTH action. However, they reported that basal ecdysone production or cAMP or MIX activation would occur in the presence or absence of calcium. That the calcium ionophore A-23187, which acts by increasing the permeability of biological membranes to divalent cations (Pfeiffer *et al.*, 1978), was able to increase the intracellular level of cAMP in *Manduca* prothoracic glands suggests that calcium and cAMP have a sequential role in PTTH activated steroidogenesis (Smith *et al.*, 1985). Smith and Gilbert (1986) have proposed that PTTH acts by increasing the influx of extracellular calcium, which in turn enhances the formation of cAMP by a calcium ion sensitive adenylate cyclase system. The cAMP may then activate a cAMP dependent protein kinase responsible for the phosphorylation of a rate limiting enzyme in the synthesis of ecdysone.

Ring glands taken from post-feeding *Calliphora* larvae were subjected to similar treatments to those from *Sarcophaga*, i.e. PTTH and dibutyryl cAMP,

and similar responses obtained. It is of particular interest that *Calliphora* ring glands responded to a crude extract of PTTH from *Sarcophaga* brains. Similar interspecies cross reactivities have been demonstrated between *M. sexta* and *S. bullata* (Roberts and Gilbert, 1986) and between *M. sexta* and mosquitoes (Gilbert, pers comm). However, although crude brain extracts are effective in the latter case, purified preparations are not. It is interesting that in the case of *Manduca/Sarcophaga* PTTH cross reactivity (Roberts and Gilbert, 1986), a degree of stage specificity was noted. When prothoracic glands or ring glands were challenged with interspecific PTTH extracts from a stage different from that of the gland donor, no dose response of gland activity was observed. These workers proposed that the ratio of the two forms of PTTH may be in some way involved in this phenomenon. O'Brien *et al.* (1986) have demonstrated that in *Manduca sexta*, the ratios change during development, and Bowen *et al.* (1984a) have shown that the sensitivity of the prothoracic glands changes depending upon developmental state. Agui *et al.* (1983) determined that PTTH/ prothoracic gland cross reactivity existed between three species of Lepidoptera, the cabbage army worm *Mamestra brassicae*, the silkworm *Bombyx mori* and *Manduca sexta*. Prothoracic glands from *Manduca* were more sensitive to the PTTH from other species and *Bombyx* PTTH was the most effective at stimulating the prothoracic glands of the other species. Despite the above qualifications, it seems probable that the mechanisms of PTTH action are very similar across a wide range of insect species and orders.

It would appear that ring glands from diapausing *Sarcophaga* have lost the ability to respond to PTTH via the calcium influx mechanism or to cAMP. Similar phenomena have been reported for *Manduca sexta* (Bowen *et al.*, 1984a; Smith *et al.*, 1986). The glands do not synthesize ecdysone at the basal levels described above. Instead, the concentrations of the hormone found in the

incubation medium were close to the lower limit of detection of the radioimmunoassay used, considerably below unstimulated larval ring gland basal levels. These results strongly support Smith *et al.* (1986) who demonstrated that diapausing *Manduca* prothoracic glands did not respond to agents which increased the intracellular levels of cAMP (MIX, dibutyryl cAMP, A23187). Interestingly, diapausing *Manduca* glands accumulated more cAMP in response to PTTH or A23187 stimulation than non-diapausing glands. This is in remarkable accordance with studies on mammalian endocrine systems which suggest that peptides that exert a trophic effect on their target tissues appear to do so by a maintenance of enzyme activity beyond adenylate cyclase. In the absence of the trophic factor, in this case PTTH, the activity of such enzymes is reduced leading to an increased production of the secondary messenger. However, these workers did suggest that cAMP synthesis may also be altered by the regulation of the adenylate cyclase system. They suggest that the absence of PTTH during the first few days of pupal life as *Manduca* enters diapause leads to decreased steroidogenic capacity accompanied by enhanced cAMP formation. The cellular basis of the loss of competency to respond to PTTH therefore occurs after cAMP production, possibly at the level of the cAMP dependent protein kinase which serves to regulate the steroidogenic process. They noted that protein kinase activity in diapausing glands is 30-40% lower than in non-diapausing glands. It was seen in chapter 3 that the levels of circulating ecdysteroids in diapausing *Sarcophaga* pupae were very low. This may be partially due to the refractory nature of the ring glands in diapause demonstrated in this study.

Ring glands from diapausing *Calliphora* larvae continue to synthesize basal levels of ecdysone. Even so, the glands become refractory to PTTH and dibutyryl cAMP stimulation. This implies that steroidogenic control may

become uncoupled from PTTH/cAMP mediation in diapause. A prothoracicotropic factor distinct from PTTH, such as that proposed by Watson *et al.* (1985), may be involved which sustains ecdysone synthesis without associated cAMP formation (Smith *et al.*, 1986). Despite the higher basal rate of synthesis of ecdysone in diapausing *Calliphora* ring glands *in vitro*, the haemolymph ecdysteroid titre is comparable with that of *Sarcophaga*. The ecdysone metabolism and excretion pathways may therefore still be in operation during diapause in *Calliphora*. Since *Calliphora* diapauses as larvae, the excretion of ecdysteroid metabolites may be possible, whereas in *Sarcophaga*, a closed system pupal diapause prevents this. To avoid an accumulation of ecdysteroids and their metabolites, basal synthesis in *Sarcophaga* may therefore be reduced. It is possible however that the *in vitro* culturing of *Calliphora* ring glands may have removed the influence of a humoral inhibiting factor which *in vivo* would have reduced the basal level of synthesis during diapause. Sehnal *et al.* (1981) demonstrated that JH can suppress the spontaneous activation of prothoracic glands and restrain the function of active glands in the waxmoth *Galleria mellonella*. The presence of elevated JH titres during larval diapauses, and its effect on PTTH release have been discussed in chapter 3. It is possible that JH is the missing factor in the incubation medium used with *Calliphora* ring glands during diapause in this investigation. Were it to be introduced, the basal levels may be reduced nearer to those of *Sarcophaga*.

Larvae of *C. vicina* have previously been described as being in diapause if they have not pupariated within 32 days of the hatching of the larvae at 12.5°C (Vinogradova and Zinovjeva, 1972). The results for ring gland competency presented here suggest that these larvae enter diapause between day 22 and day 28 post oviposition. This is unlike the rapid loss of *Sarcophaga*

ring gland competency. *Calliphora* larvae do not enter diapause at an easily identifiable stage like *Sarcophaga* pupae which do so after the formation of the phanerocephalic pupa (Fraenkel and Bhaskaran,1973). This coupled with the physiological asynchrony within the *Calliphora* population after 22 days may account for the wider time course of ring gland competency changes in *Calliphora*. It would appear that the 32 day criterion as suggested by Vinogradova and Zinovjeva (1972) for larval diapause would account for the majority of individuals at these temperatures.

This investigation showed that temperature increases administered to isolated *Calliphora* brain-ring gland complexes *in vitro* would not restore ring gland competency to PTTH stimulation; only the warming of the whole animal would do this. Smith *et al.* (1986) proposed that recovery from diapause may be dependent upon the release of trophic factors (e.g. PTTH) that ultimately stimulate steroidogenesis. However, if PTTH were the required trophic factor for recovery, one may have expected the *Calliphora* brain-ring gland complexes to recover after warming *in vitro*. Smith *et al.* (1986), however, also proposed that ecdysone synthesis may be reinitiated through the action of a haemolymph prothoracicotropic factor (Watson *et al.*,1985), apparently similar to a factor produced by the *Manduca* fat body in response to juvenile hormone stimulation (Gruetzmacher *et al.*,1984a,b). In the cotton bollworm *Heliothis*, PTTH is released in diapausing animals, but the prothoracic glands remain inactive at low temperatures and only become active as the temperature increases (Meola and Adkisson,1977; Browning,1981). It is suggested that rather than responding directly to increased temperature, the glands are activated by a temperature- sensitive increase in the levels of a humoral factor (Meola and Gray,1984). Gray *et al.* (1986) demonstrated that this factor was present only in the fat body in diapausing *Heliothis zea*. However, after a temperature rise



able to break diapause, the haemolymph titre of the factor was shown to rise considerably suggesting an association with prothoracic gland recovery. A similar non-cerebral factor may therefore be involved in this recovery in *Calliphora*. Alternatively, the temperature sensing apparatus may be located outside the brain-ring gland complex therefore requiring the rest of the body for complete ring gland recovery. The interruption of diapause in *S. crassipalpis* by the injection of cGMP and its derivatives (Denlinger and Wingard, 1978) may be involved in this process of recovery although cGMP has little steroidogenic effect on isolated *Sarcophaga* and *Manduca* prothoracic glands. Using Hexane as a tool for breaking diapause in *S. crassipalpis* (Denlinger, 1980), brains and ring glands assayed for cAMP and cGMP for the 24 hours following this treatment showed no increase in the titre of either nucleotide (Gnagey and Denlinger, 1980). Only after the ecdysone titre had risen could an increase in brain/ring gland cAMP be detected. The whole body homogenate cGMP titre however rose for three days after the hexane stimulation. They suggest therefore that there may be an interaction between ecdysteroids and cGMP in non-endocrine tissues. Evidence now points to the suggestion that although diapause induction may be a brain centred event, diapause termination is not. The involvement of the rest of the body appears to be necessary and is possibly associated with the action of cGMP and a non-cerebral factor, perhaps that suggested by Watson *et al.* (1985).

Bowen *et al.* (1984a) suggested that diapause in *Manduca* resulting from the failure of the prothoracic glands to secrete ecdysone may be in response to both the development of refractoriness to PTTH stimulation and to the curtailment of PTTH release, as opposed to its synthesis and transport. This view is supported by the data presented here from *Sarcophaga* which show that prediapause and day 35 post-pupariation diapausing pupal brains contain

comparable amounts of PTTH to non-diapause destined prepupal brains. These levels would presumably be adequate for normal adult development to continue were the PTTH released, and the ring gland competent. It appears that PTTH is present in the brains of most pupal diapausing species during diapause. In one species, *Hyalophora cecropia* (Williams, 1968) PTTH has been shown to be absent during diapause; it must be synthesized prior to recovery from diapause.

In conclusion, it would appear that ring gland function in *Sarcophaga argyrostoma* and *Calliphora vicina* is under the control of a similar PTTH activation mechanism. This probably works via a calcium dependent cAMP mediated secondary messenger system. Ring gland competency to produce ecdysone is impaired in diapause in both species apparently at a level beyond cAMP action. Diapausing *Sarcophaga* ring glands do not appear to secrete ecdysone, but those from diapausing *Calliphora* continue to secrete basal levels independently of the PTTH/cAMP system. The time course of these competency changes differs between the two species. In *Sarcophaga*, diapause, or the change in competency, appears to be more closely associated with a particular developmental stage than in *Calliphora*. Ring glands from diapausing *Calliphora* and *Sarcophaga* will recover their competency to respond to PTTH stimulation very rapidly following a temperature increase to 25°C. Denlinger (1981) suggested that diapause termination in *Sarcophaga* is a two part process, consisting of a temperature insensitive stage followed by a temperature sensitive stage in which the pupae quickly respond to a rise in temperature. The levels of PTTH in prediapause and diapausing brains of *Sarcophaga* are similar to those in non-diapause destined pupal brains. Diapause in these two species therefore results from a combination of developing refractoriness to PTTH activation by the ring glands, and a

cessation of PTTH release by the brain, though not of its synthesis and storage. Diapause termination however, can be brought about by environmental changes which may restart the release of PTTH and reverse the refractory nature of the ring gland. PTTH appears not be involved in the actual initiation of development from the diapause state; a non-cerebral factor, possibly a peptide associated with the action of cGMP, may be important. The data suggest that despite similarities in the mechanisms of diapause in the two species, there may be differences in the depth of diapause experienced by *Sarcophaga* and *Calliphora*. This is suggested by the basal levels of ecdysone synthesis in diapausing *Calliphora* larvae and by the ease of recovery by these larvae when compared with diapausing *Sarcophaga* which function within the constraints of separate temperature insensitive and temperature sensitive stages.

This study which has demonstrated that great similarities exist between the two species investigated here, both Dipteran, and the Lepidopteran *Manduca* provides evidence for common mechanisms of PTTH action and the development of diapause across a wide range of insect species. Denlinger (1981) concluded that perhaps it should not be expected that diapause in flies be identical to that in moths, and that since diapause has evolved many times, the regulating mechanisms may likewise reflect an interesting diversity. This present study suggests that this diversity may be less than Denlinger proposed.

### Discussion

Many insect species possess the capability to enter a diapause state in response to impending adverse environmental conditions. In this state, which can occur at a number of developmental stages in different species, the growth and development of the individual or its reproductive system is arrested and the level of metabolic activity is reduced. The onset of diapause in many species is controlled by photoperiod such that, in the case of long-day species, such as *Sarcophaga argyrostoma* and *Calliphora vicina*, diapause is induced by the shortening daylength of autumn and winter.

Diapause in *S. argyrostoma* is primarily induced during the embryonic and early larval stages of development. Despite these stages being the most photoperiodically sensitive, there appear to be few differences between growth and developmental rates in diapause and non-diapause destined cultures. Prior to ovulation, no photoperiodic sensitivity was noted, a finding in agreement with those of Bradley and Saunders (1985). When embryos were removed from the females soon after ovulation and exposed to a variety of photoperiodic regimes, identical responses (in terms of diapause incidence) to those left *in vivo* were obtained indicating the absence of any form of maternal influence. Despite the fact that diapause is induced most strongly at these stages, no evidence of diapause destiny was noted until after the formation of the phanerocephalic pupa aside from an increase in the length of the larval wandering period in the short-day culture. The implication of this is that photoperiodic 'information' acquired during early development is stored for later 'consultation'. The most likely candidate for this form of information

storage may be that of a 'diapause titre' as proposed by Gibbs (1975). He suggested that successive long-nights encourage the formation of a titre which is then stored. This titre could then be compared with an internal threshold. If the titre is above the threshold, then diapause would result, if not, then development would continue uninterrupted. Goryshin and Tyshchenko (1974) proposed a similar model in which daylength (or nightlength) is measured by an 'oscillator link' (clock) only capable of the qualitative distinction of long and short nights. This information is stored in discrete information packets by a thermostable 'memory link' (counter). Comparison to a threshold value controls a 'neuroendocrine link' which then directs subsequent development. If these explanations are to be considered valid, they must be adapted to account for the effects of temperature and crowding. When cultures are exposed to short-days at high temperatures, the diapause incidence is less than that at lower temperatures (Saunders, 1971). If the diapause titre is unstable, the rate of decay may be accelerated at high temperatures thereby bringing the titre below that of the internal threshold. Alternatively, since development occurs more rapidly at high temperatures, the insects will experience fewer short-days before they lose their photoperiodic sensitivity late in larval development. Less titre will be accumulated and diapause incidence will be lower. It is possible that a combination of these two factors may account for the effects of temperature. When post-feeding larvae are maintained under crowded conditions, pupariation is delayed. However, those crowded larvae which pupated early in the group were found to display a low diapause incidence when reared under short-day conditions. Uncrowded larvae which pupariated late in the group, and therefore experienced the same number of short-days as the crowded early group, had a high diapause incidence. Prior to their having left the food, both groups were

kept under identical conditions and presumably were equally diapause destined. The mechanism by which diapause incidence can be altered is not known but must, if the titre theory is correct, lie either with a reduction in the effect of the titre or a raising of the internal threshold, possibly as a result of excessive contact with, or proximity to other wandering larvae. Since uncrowded larvae were kept in tubes stacked inside light tight boxes, the existence of an air borne chemical messenger seems unlikely. Bradley (1984) demonstrated that the enclosure of post-feeding *Sarcophaga* larvae in tight, clear plastic tubing, such that they were always in contact with the walls of the tube, both delayed pupariation and lowered diapause incidence. This worker also showed that solitary larvae in the vicinity of a crowded culture were not affected, either in terms of pupariation time or diapause incidence, by this close proximity thereby ruling out the existence of a pheromone type messenger. Physical contact with other larvae therefore seems most likely as a cause of delayed pupariation and changing diapause incidence.

The major question to be posed by this section is how photoperiodic information is stored, *i.e.* where and what is the diapause titre. The brain would seem to be the most likely candidate for the location of the mechanism in *Sarcophaga* since it has a pivotal role in most other developmental events. The photoperiodic clock/counter mechanism appears to be located in the brain in the tobacco hornworm *Manduca sexta* (Bowen *et al.*, 1984b). These workers reversed the diapause commitment of short-day reared *Manduca* larvae by the implantation of brains from larvae that had previously received long-day stimulation. In addition, they were able to programme brains by exposing them to photoperiods whilst *in vitro*. This provided good evidence for the location of the whole photoperiodic receptor/clock/counter/effector mechanism in the brain. Similar results were obtained by Giebultowicz and Saunders (1983) who

reversed diapause commitment in *Sarcophaga argyrostoma* by the implantation of long-day brain/ring gland complexes (programmed *in vivo*) into short-day larvae 3 days after wandering. Cells responsible for the secretion of the neuropeptide prothoracicotropic hormone (PTTH), the absence of which is implicated in diapause, are located in the brain, apparently with axonal connection to close to the prothoracic glands (Agui *et al.*,1979; Giebultowicz and Denlinger,1986). The position of the mechanism within the brain would be harder to ascertain. If the titre is stored in a small number of cells, it may be possible to determine their position by transplanting portions of diapause and non-diapause programmed brains into non-diapause and diapause destined individuals to see if their developmental fate can be altered. However, if such cells require direct neural connection to effector mechanisms such as the PTTH neurosecretory cells (NSCs), such experimental procedures may not produce results. Williams (1969) demonstrated that the interruption of normal interneural transmission by tetrodotoxin did not affect the photoperiodic responses of pupal *Antheraea pernyi*. In this case, it is possible that the photoperiodic mechanisms regulating PTTH release reside within the PTTH neurosecretory cells themselves.

The induction of diapause by photoperiod is controlled via the circadian clock system in *Sarcophaga*, as in many other insect species (Saunders,1982). Pittendrigh and Minis (1964) proposed an external coincidence model to account for this photoperiodic response in which a particular phase of an endogenous rhythm coincided with a phase the light dark cycle. In *Sarcophaga*, if a photoinducible phase, which occurs 9.5h after the onset of the dark period is illuminated, diapause is not induced (Saunders,1975). However, if the photoinducible phase falls within the dark period (*i.e.*, the insect is subjected to a long night), diapause titre is accumulated. A series of these long nights is

required to accumulate sufficient titre to cross the threshold proposed by Gibbs (1975) and induce diapause. The circadian basis of photoperiodic diapause induction was confirmed by Saunders (1976) using night interruption experiments in which 1 hour light pulses scanned the greatly extended dark period in a light-dark cycle of 72h. The photoinducible phase was found to occur with a circadian rhythmicity of about 25h. Were it not a circadian based system, one would have expected only one photoinducible phase, 9.5h after lights-out, to have occurred. The clock in this case therefore measures nightlength rather than daylength.

The present work has shown that PTTH release can be directly controlled by the circadian system since larval exodus from the food, which is initiated by a burst of PTTH release, is a gated event. The circadian clock is set by the photoperiodic regime in which the embryos and larvae are raised therefore, the storage of diapause inducing titre may be closely associated with these clock and endocrine effector mechanisms. It may even be possible that the titre is stored within the PTTH NSCs themselves and that following the release of PTTH at pupal apolysis, its influence is exerted. Presumably, the cells containing the titre must be in contact with the mechanisms, possibly touch receptors, which account for the effect of crowding. Such contact is most likely to be neural, at least in part. It could either take the form of direct neural pathways to the brain or via a neurosecretory cell/hormone system which produces an antagonistic effect to titre accumulation/expression, or raises the internal threshold.

The identity of the titre will not be easy to determine, since to date, no compositional differences have been found between diapause and non-diapause destined brains. It has been shown however that the brains of short-



day reared *Sarcophaga* are in some way qualitatively different from those which were raised in long-days in that the implantation of non-diapause destined brains into diapause destined larvae can avert entry into diapause (Giebultowicz and Saunders, 1983), whereas in the reverse experiment, short-day brains cannot induce diapause when implanted into long-day animals. Extracts of brains from wandering larvae or pupae (to increase the chances of a detectable quantity) from both lines should be compared using a number of different analytical techniques. If it is peptidaceous or proteinaceous in nature, and present in sufficient quantities, electrophoretic techniques may be able to detect its presence. Alternatively, chromatographic techniques may help. By a combination of extraction, separation and detection procedures, some idea of the identity of the titre may be obtained. If the titre exists, and is isolated, the position in the brain may be more closely determined if antibodies can be raised against it. Immunohistochemistry could be used to locate the region in which it is stored. It should be stressed that since no hard evidence for the existence of such a titre is available, these ideas should be regarded as wholly speculative.

Larvae and pupae raised in both long and short-days display few differences in their haemolymph ecdysteroid titres up to the formation of the phanerocephalic pupae, aside from a higher titre associated with a shorter wandering period in non-diapause destined cultures. That diapause has been induced apparently has little effect on the general endocrine sequences involved in larval and early pupal development. This would agree with the ideas raised above that diapause destiny is reversible up to a late stage by temperature/crowding effects. Were differences noted earlier, reversibility would presumably be harder to achieve since diapause would not just be a brain centred 'decision', but would also have involved the development of

peripheral tissues. This is not to say that entry into diapause can be achieved by the brain alone. The rest of the body may still be required. After the formation of the phanerocephalic pupae, those pupae raised in short-days apparently undergo an endocrinological shut-down. The haemolymph ecdysteroid titre falls to close to the limit of detection whereas those continuing uninterrupted development display high titres. The concept of this shut-down is not a new one, having first been proposed by Wigglesworth (1934,1936). Williams (1946,1947,1952) further developed the idea by suggesting that in diapause, the brain failed to release a factor required for development. This factor is now known to be PTTH. The present investigation has shown that this hormone is present in large quantities in pupal non-diapause destined, pre-diapause and late diapausing brains of *Sarcophaga*. It seems likely therefore that its release is indeed curtailed in diapause in this species. In addition to this system of preventing the synthesis of ecdysone and thereby restricting development, the prothoracic glands in both diapausing *Sarcophaga* and *Calliphora vicina* are not able to respond to PTTH stimulation *in vitro*. Differences do exist between the two species in that diapausing *Sarcophaga* prothoracic glands do not synthesize and release any detectable ecdysone whereas those from *Calliphora* continue to synthesize at pre-diapause basal levels. This basal synthesis cannot be elevated by PTTH or cyclic AMP stimulation as it can in non-diapausing prothoracic glands. The ecdysteroid synthesis mechanisms appear to be intact, but uncoupled from the normal hormone/secondary messenger mediating mechanism. Whether it can be affected by externally (to the prothoracic gland) applied agents remains to be seen. The current suggestion in the literature is that ecdysone synthesis is controlled via a cAMP dependent protein kinase responsible for the phosphorylation, and therefore activation of ecdysteroidogenic enzymes. The

block in prothoracic gland competency in diapause is believed to exist at the level of this protein kinase (Smith *et al.*, 1986). Evidence presented here would appear to support these suggestions. However, the *Calliphora* diapause situation implies that either steroidogenesis can be self sustaining, or that a second hitherto undetermined mechanism exists outside the PTTH/cAMP/protein kinase system. It is interesting to note that ecdysone produced during diapause in *Calliphora* apparently does not accumulate in the haemolymph. Presumably therefore, metabolic and/or storage/excretion pathways are still operative.

Evidence presented here appears to suggest that entry into diapause may be a brain centred event whereas recovery, whilst obviously involving the brain/ring gland complex in *Calliphora*, does require that it be *in situ*. This recovery therefore may not be a simple reversal of the endocrinological shutdown of diapause entry, with PTTH release bringing about the resumption of hormonal activity. The order in which recovery is achieved is not known. A humoral factor, as discussed in chapter 4, may act upon the brain causing PTTH release to resume which then brings about ring gland competency or, the factor may act directly on the ring gland causing the synthesis of ecdysone which then feeds back onto the brain to restore the release of PTTH. It may be possible to test these options by incubating diapausing brain/ring gland complexes or ring glands alone in haemolymph from recovering *Calliphora* larvae. Whilst it is suspected that similar mechanisms exist in the recovery from pupal diapause in *Sarcophaga*, it would be easier to use *Calliphora* as the vehicle for testing these hypotheses because of the relative ease of removing brains and ring glands from larvae as opposed to pupae.

One of the most important questions to be suggested by the present work

is how is PTTH release regulated, both in normal development and in diapause? The release of PTTH prior to a moult is thought to occur as a result of proprioceptor stimulation. Wigglesworth (1933) noted that abdominal distension was required to stimulate a moult in the bloodsucking bug *Rhodnius prolixus* and concluded that proprioceptive stimuli controlled PTTH release in this species. Since proprioception is a nervous phenomenon, it is possible that this system is mediated by the sensory nervous system. Wigglesworth (1934) confirmed this by sectioning the ventral nerve cords immediately after feeding. These animals failed to elicit a moult. Orchard and Steel (1980) demonstrated that stretch receptor stimulation initiated electrical activity in the medial NSCs of *Rhodnius*, and that this occurred simultaneously with changes consistent with a sudden onset of transport and release of neurosecretory material. Steel *et al.* (1982) further noted that a few hours later, the haemolymph ecdysteroid titre increased, presumably in response to stimulation of the prothoracic glands. Injury is another form of proprioception which appears to affect PTTH release. O'Farrell and Stock (1953) demonstrated that the removal of a limb from the larva of the cockroach *Blattella germanica* delayed moulting until the limb had regenerated. It has been proposed that injury may result in the activation of the corpus allatum, the site of juvenile hormone synthesis (Bollenbacher and Granger, 1985). If this is the case, the proposed mechanism for JH inhibition of PTTH release in larval *Manduca* (Nijhout and Williams, 1974) could be functioning in this response.

Whilst the above observations suggest that PTTH release can be stimulated or delayed by the peripheral tissues, they fail to explain the mechanisms by which PTTH release is controlled. Little is known about the regulation of neurosecretion in insects. The following sections will address this question,

which will become increasingly important in the future, in more detail. It should be stressed that in the absence of any extensive documented evidence in insects, much of the following is speculative. Where possible, parallels with the more widely studied mammalian systems will be drawn.

Morphine and the endogenous opioid peptides (including  $\beta$ -endorphin, methionine-enkephalin and leucine-enkephalin) exert similar effects on the mammalian neuroendocrine system (Meites *et al.*, 1979). When administered in acute doses into rats, they stimulate the release of growth hormone, prolactin and adrenocorticotropin into the serum. They also inhibit the release of luteinizing hormone, follicle stimulating hormone and thyrotropin. Naloxone, an opioid antagonist (Beaumont and Hughes, 1979), when injected into rats reversed all the above effects. This naloxone reversibility is considered a prerequisite of an opiate/opioid system. The opioid peptides appear to act mainly by inhibiting neuronal activity. The release of adenohypophysial hormones (those from the anterior lobe of the pituitary gland) may therefore be due to a blocking of the inhibitory effects of release inhibiting factors, or via dopaminergic or serotonergic mechanisms, which are thought to control the releasing factors. Romeuf and Remy (1984) have identified met-enkephalin-like neuropeptides in two groups of NSCs in the brains of adult locusts, *Locusta migratoria*. Enkephalin-like material has also been identified by Gros *et al.* (1978) in the optic lobes and corpora cardiaca of the same species. Stefano and Scharrer (1981) demonstrated the existence of high affinity binding sites for an enkephalin-like ligand in the cerebral ganglion of the cockroach *Leucophaea maderae*. They concluded that opioid receptors and therefore endogenous opioid peptides are present in the nervous system. De Loof and co-workers (1986) have presented preliminary results that suggest the presence of a wide range of neuropeptides in insect brains, including those of

*Sarcophaga bullata*. The physiological role of these peptides in insects is not known, although Romeuf and Remy (1984) suggest a neuromodulatory effect of met-enkephalin-like peptides on the production of neurosecretory material from the *pars intercerebralis*, the area of the brain containing the putative PTTH NSCs. These workers further demonstrated the presence of the tetradecapeptide somatostatin in locust brains. This peptide has been found to inhibit the secretion of growth hormone from rat pituitary incubates *in vitro* (Krulich *et al.*, 1968).

This indirect evidence suggests that these peptides may be involved in the regulation of PTTH production and/or release. Richard and Saunders (unpublished data) suggest that morphine and naloxone may affect PTTH release. Using brain/ring gland complexes from wandering larvae *in vitro*, which produce a low basal level of ecdysone, 10mM morphine sulphate in the incubation medium can raise ecdysone production 10 fold. Conversely, brain/ring gland complexes from red spiracled larvae, which show a higher basal level of ecdysone synthesis because they are presumably already being subject to PTTH stimulation, show a marked reduction when incubated with naloxone. Although the concentration of morphine required is high, possibly because of problems of entry into the central nervous system or differences in receptors, these preliminary observations meet some of the criteria thought necessary to conclude that opioid peptides play a role in the regulation of PTTH release. Future studies in this area should concentrate on the effects of various opioids (met- and leu-enkephalin and  $\beta$ -endorphin), other peptides, for example somatostatin, and their agonists and antagonists, on PTTH release. These experiments may indicate whether neuropeptide regulation of PTTH release is a natural phenomenon in insects, and should lead to further investigation of their concentration and distribution within the brain.

It is suggested that somatostatin should receive particular attention from future investigators. Rodbell (1980) proposed that this widely occurring peptide acts by two possibly interrelated mechanisms: by inhibiting the formation of cAMP and by inhibiting calcium entry into the cell. Many neurotransmitters exert their action by modulating adenylate cyclase action. This calcium/cAMP-mediated system is known to exist in the prothoracic glands of some insect species (see chapter 4). It may also be involved further upstream in the control of PTTH NSCs. Rasenick *et al.* (1978) noted increases in brain cAMP levels associated with recovery from diapause in the giant silkworm *Antheraea pernyi*, as measured by the reactivation of neurosecretion. It is possible that during diapause in this and possibly other species, somatostatin acts by blocking cAMP mechanisms which may be involved in PTTH release. Recovery may therefore involve the removal of the somatostatin block. Observations by Gnagey and Denlinger (1983) that *S. crassipalpis* pupae recovering from diapause do not accumulate cAMP in the brain suggest that this mechanism may not be operative in all species. However, if somatostatin is specifically targeted to the PTTH NSCs, cAMP levels may have been below detectable levels.

A second group of neuromodulatory hormones that may be involved in PTTH release are the biogenic amines. Whilst endogenous opioids may act as neurotransmitters in their own right, Meites *et al.* (1979) have suggested that they may act by affecting serotonin and dopamine metabolism. In mammals, serotonin can increase prolactin and growth hormone release from the pituitary gland, whereas in some cases, dopamine has been shown to be inhibitory. Samaranayaka (1976) has presented evidence to suggest that the release of adipokinetic hormone from the corpora cardiaca of the locust *Schistocerca gregaria* is reduced by a depletion of monoamines in the nervous

system. She suggests that the regulation of release is controlled by a composite serotonin/dopamine receptor. Another biogenic amine,  $\gamma$ -aminobutyric acid (GABA) has been implicated in the neuromodulation of hormone release. Gibbs (1984) showed that picrotoxin, a GABA antagonist, can avert pupal diapause in *Manduca* by causing PTTH release, whilst in *Mamestra brassicae* (Agui, unpublished), the *in vitro* culturing of brains and prothoracic glands has shown that acetylcholine stimulates PTTH release, but that GABA inhibits it.

The involvement of adenylate cyclases sensitive to serotonin and, to a lesser extent dopamine and octopamine has been proposed in brain homogenates of the saturniid moths *Hyalophora cecropia* and *A. pernyi* (Rasenick and Berry, 1981). Activation of adenylate cyclases may be involved in diapause termination in these species, presumably through an effect on the release of PTTH. There is substantial evidence that the neurosecretory somatostatin neurones in the rat hypothalamus receive a catecholaminergic (probably dopaminergic) input (Arimura and Fishback, 1981). Thus in rats, dopamine stimulates the release of somatostatin which then inhibits the release of growth hormone (GH). However, circulating GH levels could also be increased by the application of L-dopa and of other dopaminergic agonists. These apparently paradoxical results suggest that both somatostatin and growth hormone releasing hormone (GHRH) may be regulated by dopaminergic stimuli, and that the effects of dopaminergic factors on pituitary GH secretion may reflect a delicate balance between the effects of the release of these two factors. Since GHRH acts by increasing adenylate cyclase activity (Rodbell, 1980), a parallel with the saturniid moth system may become apparent with dopaminergic factors being able to both stimulate adenylate cyclase activity (thereby releasing PTTH, and breaking diapause), and possibly produce



the inhibitory factor somatostatin.

Using the brain/ring gland preparations from *Sarcophaga* and *Calliphora in vitro*, it should be possible to test various neurotransmitters (acetylcholine, GABA, octopamine, dopamine, serotonin *etc.*) together with their agonists and antagonists, in a pharmacological study of PTTH release. Initially, such studies should concentrate on the wandering and red spiracled stages of larval development to determine their modes of action, *i.e.* stimulatory or inhibitory. Future workers should be directed to a comprehensive review of biogenic amines in the insect nervous system by Evans (1980) and to volume 11 of Comprehensive Insect Physiology, Biochemistry and Pharmacology (Eds. Kerkut and Gilbert).

A third factor that is apparently involved in the regulation of PTTH secretion is juvenile hormone. Nijhout and Williams (1974) reported that the decline in JH titre at pupation in *Manduca sexta* was the permissive factor for PTTH release at that moult. It is suggested that JH acts as a means of restraining PTTH release from commencing, rather than by turning off release that has already started. Hiruma *et al.* (1978) found that injection of a JH analogue into the larvae of *Mamestra brassicae* induced the accumulation of stainable neurosecretory material in the NSCs thought to be the source of PTTH in this species. JH therefore apparently restrains PTTH release. This effect did not occur throughout the instar, but only as the larva neared moulting, indicating a changing sensitivity of the brain to JH. Sehnal *et al.* (1981) noted that application of JH early in the instar of the wax moth *Galleria mellonella* apparently accelerates PTTH release, but later in the instar actually inhibits it. Walker and Denlinger (1980) reported that whole body homogenates of *Sarcophaga crassipalpis* displayed pulses of JH activity in diapause destined

prepupae which were absent in those destined for continuous development. These pulses occurred with a period of approximately 24h through pupation and the onset of pupal diapause. In mid-diapause, JH activity was found to correlate with metabolic activity. JH was undetectable on days of high oxygen consumption, but was present at high levels on days of reduced oxygen consumption. These workers proposed that this periodic JH activity could be used as a time-keeping mechanism that would accumulate covert effects of the hormone and thus determine the duration of diapause. That exogenous JH can greatly reduce diapause duration supports this theory. Whilst it can shorten diapause, it can not itself terminate it (Zdarek and Denlinger, 1975). It does however greatly increase the efficacy of 20-hydroxyecdysone to terminate diapause. This evidence suggests not a direct role for JH in stimulating diapause recovery, but rather an indirect role in promoting the action of 20-hydroxyecdysone, perhaps by priming the tissues to respond.

It is not intended that the above sections on the control of PTTH release should be in any way a definitive discussion of potential and established methods of regulation; rather that they may stimulate the further investigation of vertebrate mechanisms in insect endocrinology. The literature contains many references to vertebrate-like neurotransmitters and hormones present in insects but few direct roles have yet been proposed. It is likely that these factors are integrated into the classic PTTH/ecdysteroid/JH picture of insect development and diapause. It is hoped that future workers will target these research areas in order to establish just how such integration is achieved.

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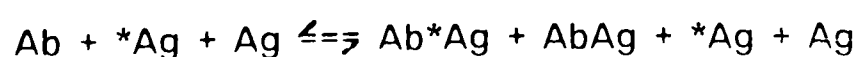
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## I. Appendix 1. The radioimmunoassay of ecdysteroids

Since Berson and Yallow (1958) first reported the development of a radioimmunoassay (RIA) for the vertebrate hormone insulin, the field of endocrinology has undergone a revolution in the technical capability of quantifying submicrogram levels of hormones. One of the major advances in the specific field of insect endocrinology was the development of an ecdysteroid RIA by Borst and O'Connor in 1972. Prior to this development, ecdysteroid assays were carried out using ligated larval abdomen bioassay techniques, either with *Calliphora* (Becker and Plagge, 1939, Adelung and Karlson, 1969) or *Musca* (Kaplanis *et al.*, 1966).

Before the methodology of the ecdysteroid immunoassays used in the present investigation are described, a brief explanation of the general principles of RIA will be presented. Normally, a fixed saturable quantity of an antibody (Ab) raised against an immunogen by injection into a suitable mammal, in this case, a rabbit, is incubated in the presence of a fixed quantity of radioactively labeled antigen (\*Ag) and a specified (in the case of a standard) or unknown (in the case of a sample) quantity of unlabeled antigen (Ag). In the present investigation, the immunogen used to raise the antisera was an ecdysteroid bound chemically to thyroglobulin, a large protein molecule. This conjugation was required to increase its immunogenicity since small molecules like ecdysone (MW 464) do not themselves elicit a good immunogenic response. After incubation of the RIA mixture, the amount of bound \*Ag therefore depends upon the amount of unlabeled antigen present in the mixture. This is demonstrated in the general reaction equation below,



where the total of \*Ag and Ag is in excess of the total number of antibody binding sites available. It is therefore a simple competition in which Ag reduces the availability of Ab to \*Ag. The greater the concentration of Ag present, the less \*Ag is bound. Incubations are generally carried out at low temperatures since this encourages the reaction to move to the right of the above equation. Once equilibrium has been reached, the bound and free fractions of labeled antigen are separated and the amount of bound radioactivity measured. The antigen concentration in an unknown sample is measured by comparing the reduction of \*Ag binding produced by Ag in the sample to that of a standard curve produced by adding known, graded amounts of Ag to the assay system.

In the present studies, two different antisera were used, Horn-2, derived from a 20-hydroxyecdysone-2-succinylthyroglobulin immunogen, capable of detecting phase 1 (oxidation) and phase 2 (conjugation) metabolites of the A-ring of ecdysone, for example 3- $\alpha$ -epimers and ester conjugates at C-2 and C-3 (Warren and Gilbert, 1986), and Horn-22, raised against an ecdysone-22-succinylthyroglobulin immunogen, which can detect ecdysone and its metabolites resulting from phase 1 and phase 2 reaction of the side chain.

Two methods of separating the bound and free \*Ag fractions were used in the present investigations. A second antibody precipitation method using donkey anti-rabbit immunoglobulin G antiserum was developed by this author in Edinburgh and was the primary method used. A similar double antibody precipitation method was reported by Lazarovici *et al.* (1983). A protein-A method developed by Warren *et al.* (1984) was also used in specified portions of the investigation. The second antibody method works by incubating the equilibrated RIA mixture with an antiserum raised against the general species

of first antibody (Horn-2 and Horn-22, both raised in rabbits). The proportions of the first and second antibody should be carefully controlled to maximize the precipitate and therefore the recovery of the radioactive antigen. If too little second antibody is used, few cross linkages are formed between the first and second antibody molecules and a weak easily disrupted precipitate is produced resulting in a low recovery. However, if too much is added, too many small accumulations are formed which can not easily be recovered by centrifugation, with the same result. At the optimum ratio, known as the zone of equivalence, large sturdy precipitates are formed which can easily be recovered. The protein-A method relies upon a suspension of formaldehyde-treated *Staphylococcus aureus* cells. Such cells are coated with protein-A which binds specifically to the Fc portion of antibody molecules. An excess of protein-A was added and the mixture centrifuged to effect the recovery of the bound fraction. In both methods, the supernatant containing the free fraction was aspirated following the centrifugation step

## Materials and methods

The first and second antibody solutions, and the radioactive ecdysone solution were all made up with borate buffer pH 8.4.

### Borate buffer

6.18 g Boric acid (0.1M) 9.54 g Borax (Sodium tetraborate) (0.1M) 4.38 g NaCl (0.075M)

Dissolve in one litre of distilled water and adjust pH to 8.4.

**Tritiated ecdysone**,  $\alpha$ -[23,24-<sup>3</sup>H(N)] (New England Nuclear) (specific activity 80.0 Ci/mmol) was obtained in 0.05mCi quantities in 0.5ml of methanol:toluene, 1:9. This was dissolved in 250ml of borate buffer containing sodium azide as a preservative, and stored at -10°C in 50ml aliquots until use. This concentration gave 18000 dpm / 100 $\mu$ l, the amount used in each assay.

**Horn-22 antibody** raised in rabbits was kindly supplied by Professor L.I. Gilbert, University of North Carolina at Chapel Hill, N.C., USA., and was diluted 2000-fold in borate buffer plus sodium azide prior to use. 100 $\mu$ l was added to each assay tube. Horn-2 antibody, also supplied by Professor Gilbert was diluted 2500-fold and again, 100 $\mu$ l added to each assay.

**Donkey anti-rabbit IgG second antibody** was obtained from the Scottish Antibody Production Unit. The concentration used was determined for each primary antibody. This will be discussed after a description of the assay protocol.

**Protein-A** was prepared in Professor Gilbert's laboratory as a 5% wet weight/volume suspension in borate buffer of formaldehyde treated

*Staphylococcus aureus* cells of the Cowan 1 strain prepared according to the instructions of Kessler (1975) as cited in Warren *et al.* (1984).

**Ecdysone and 20-hydroxyecdysone** standards were prepared in Grace's medium (Gibco) as described in the standard curve figure legends. 10µl of each standard was used to prepare the standard curve. Standards were stored at - 10°C.

The radioimmunoassay was set up with 4x non-specific binding tubes (that label bound in the absence of any first antibody), 4x maximum binding tubes (that bound in the absence of any competing sample or standard), 3x each standard concentration, and 2x each sample. 100µl of the first antibody solution was added to each tube (except the non-specific binding tubes) together with the sample or standard (except the maximum binding tubes), and 100µl of the radioactive ecdysone solution. All tubes were vortexed briefly, covered with parafilm to avoid evaporation, and incubated at 4°C overnight. 100µl of the second antibody solution was added, the tubes vortexed again, and reincubated at 4°C for 3 hours. When protein-A was used, 20µl was added and the tubes incubated for 30 minutes. Following incubation, the tubes were centrifuged at either 5000 x g for 15 minutes or 45 minutes at 2000 x g, both at 4°C, and the supernatant aspirated with a Pasteur pipette attached to a vacuum line. No difference was noted in the recovery of radioactive label between the two centrifugation protocols. 20µl of distilled water to resuspend the pellets and 500µl of Ultrafluor (National Diagnostics) scintillation fluid was added, each tube vortexed to leave a clear liquid, and the tubes counted by tritium scintillation spectroscopy.

The results obtained were treated by converting the standard concentration counts to a percentage of the maximum bound count. Both were corrected for



non-specific binding prior to the calculation. These percentages were plotted against the logarithm of the ecdysteroid concentration, and a line plotted by linear regression.

## Results

The concentration of second antibody used with each primary antibody was established by setting up a series of maximum binding tubes, allowing antigen- antibody binding to occur at the above dilutions which were recommended by Professor Gilbert. Figure A-1 shows that at low and high levels of second antibody concentration, little radioactivity is recovered from the Horn-22 assay. However, when 0.75 $\mu$ l (100 $\mu$ l of 1/150 donkey-anti rabbit IgG) is added, the maximum recovery is effected. Figure A-2 shows similar data obtained from Horn-2 maximum binding assays. Whilst the second antibody precipitation method was not used with Horn-2 antiserum in the current investigations, the data is presented to provide a confirmation of the viability of the method for its use with Horn-22. In the case of Horn-2, 0.5 $\mu$ l (100 $\mu$ l of 1/200 donkey anti-rabbit IgG) was found to be optimum. These concentrations were used in each investigation.

Figure A-3 shows the standard curve of ecdysone and 20-hydroxyecdysone with Horn-22 antibody. It can be seen that the same concentration of the two ecdysteroids effects a different inhibition of radioactive ecdysone binding. The ratio of ecdysone to 20-hydroxyecdysone binding at 50% of the maximum bound was 1.92. This antiserum does not distinguish very well between the two ecdysteroids, suggesting that it may be of use with the analysis of mixed samples, for example, haemolymph. A similar treatment of Horn-2 antiserum shows a binding ratio at 50% of maximum binding of 7.90 (figure A-4). This antiserum would not be of use with mixed samples because of the wide difference between the ecdysone and 20-hydroxyecdysone binding. It would be of more use in the analysis of samples containing predominantly one ecdysteroid, for example, those from the incubation of prothoracic glands

which produce ecdysone as the major ecdysteroid (chapter 4), or those which had been separated by chromatography (chapter 3).

When the effectiveness of the two methods of assay termination, double antibody and protein-A, were compared (figure A-5), it was seen that there was little difference between the two. The standard curves produced by these two methods with Horn-22 antiserum and ecdysone standards showed the same concentration at 50% of maximum binding.

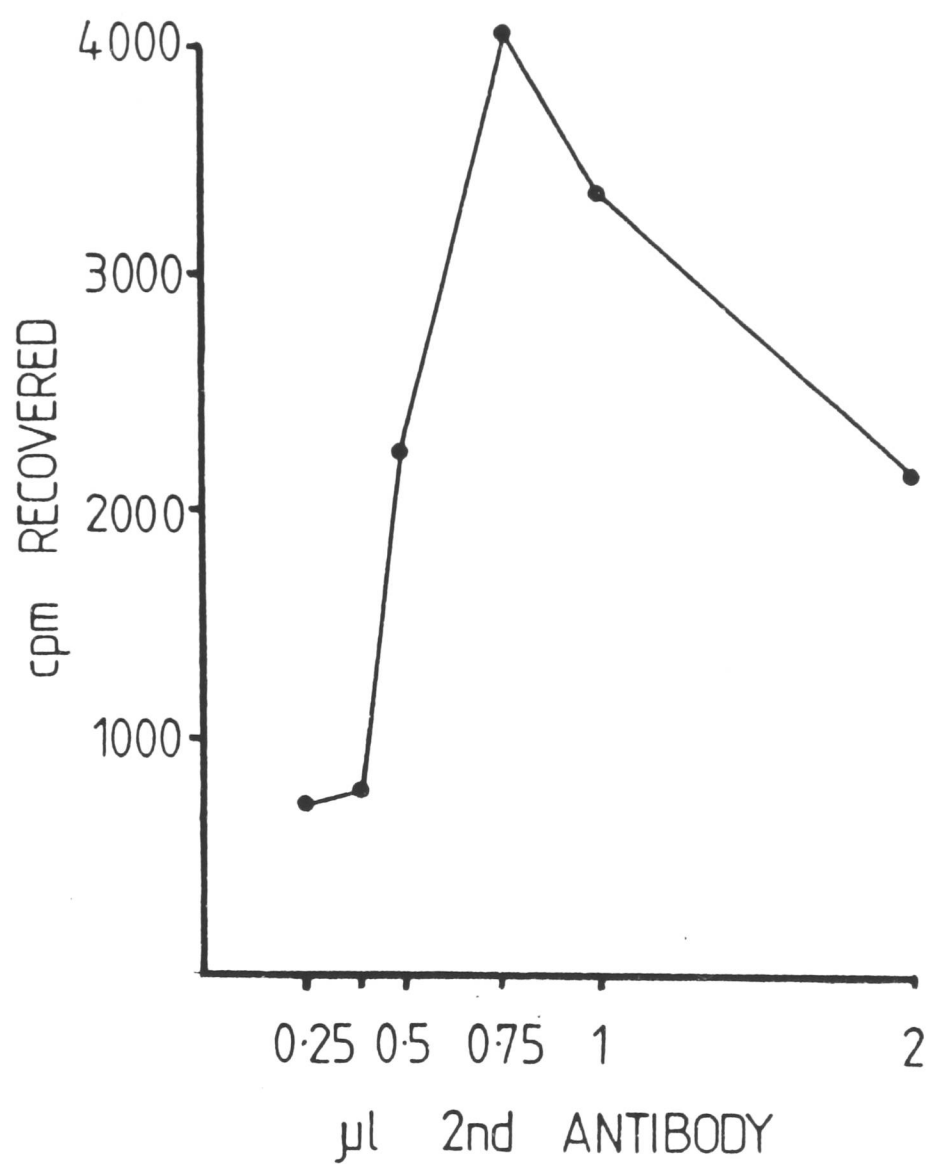


Figure A-1: The effect of varying the concentration of donkey anti-rabbit IgG antiserum ( $\mu\text{l}$  per assay, made up to 100 $\mu\text{l}$  with borate buffer) on the recovery of radioactive label (counts per minute, cpm) from the Horn-22 maximum binding radioimmunoassay. 100 $\mu\text{l}$  of 0.05% Horn-22 and 100 $\mu\text{l}$  of radioactive ecdysone (18000 dpm) were added to each tube to produce the maximum binding points. Each point represents the mean of 3 determinations.

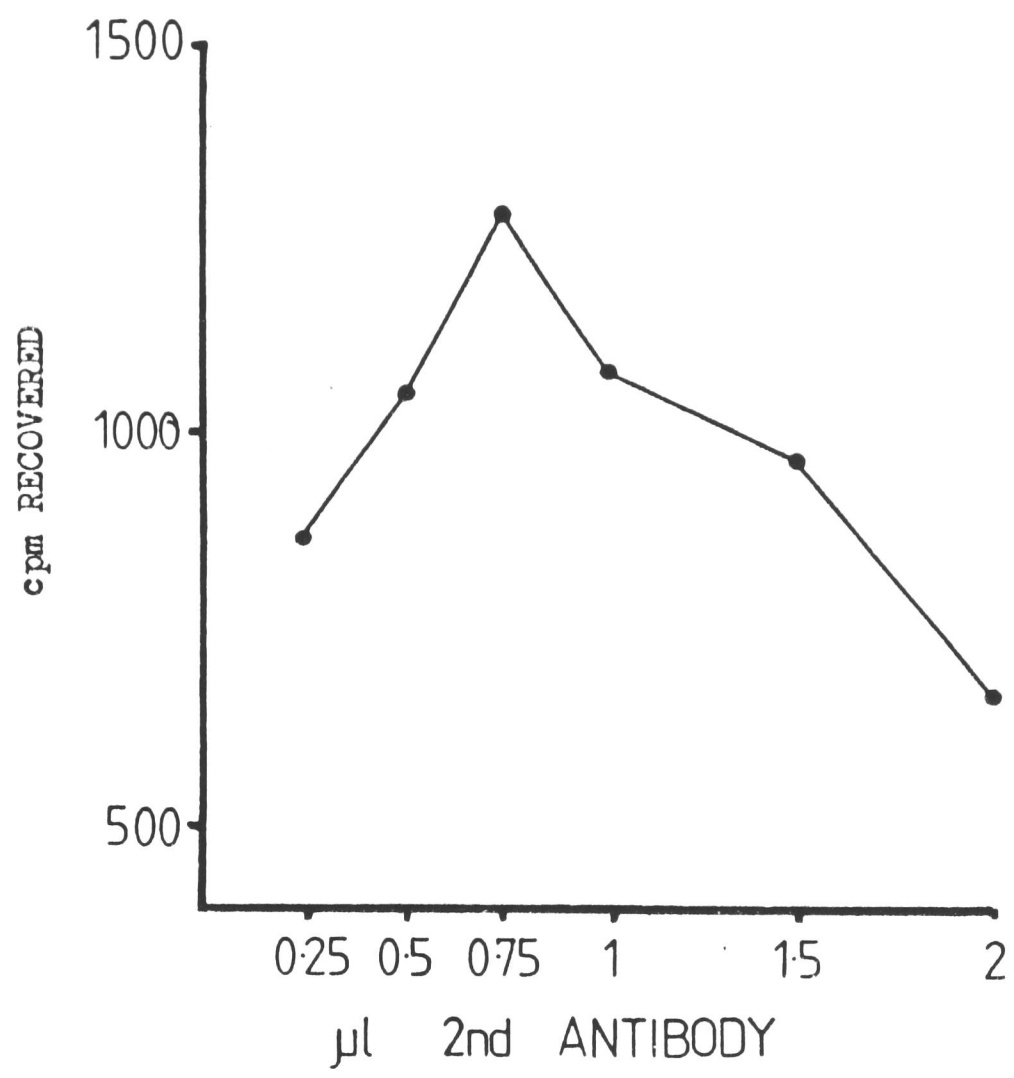


Figure A-2: The effect of varying the concentration of donkey anti-rabbit IgG antiserum ( $\mu\text{l}$  per assay, made up to 100 $\mu\text{l}$  with borate buffer) on the recovery of radioactive label (counts per minute, cpm) from the Horn-2 maximum binding radioimmunoassay. 100 $\mu\text{l}$  of 0.04% Horn-2 and 100 $\mu\text{l}$  of radioactive ecdysone (18000 dpm) were added to each tube to produce the maximum binding points. Each point represents the mean of 3 determinations.

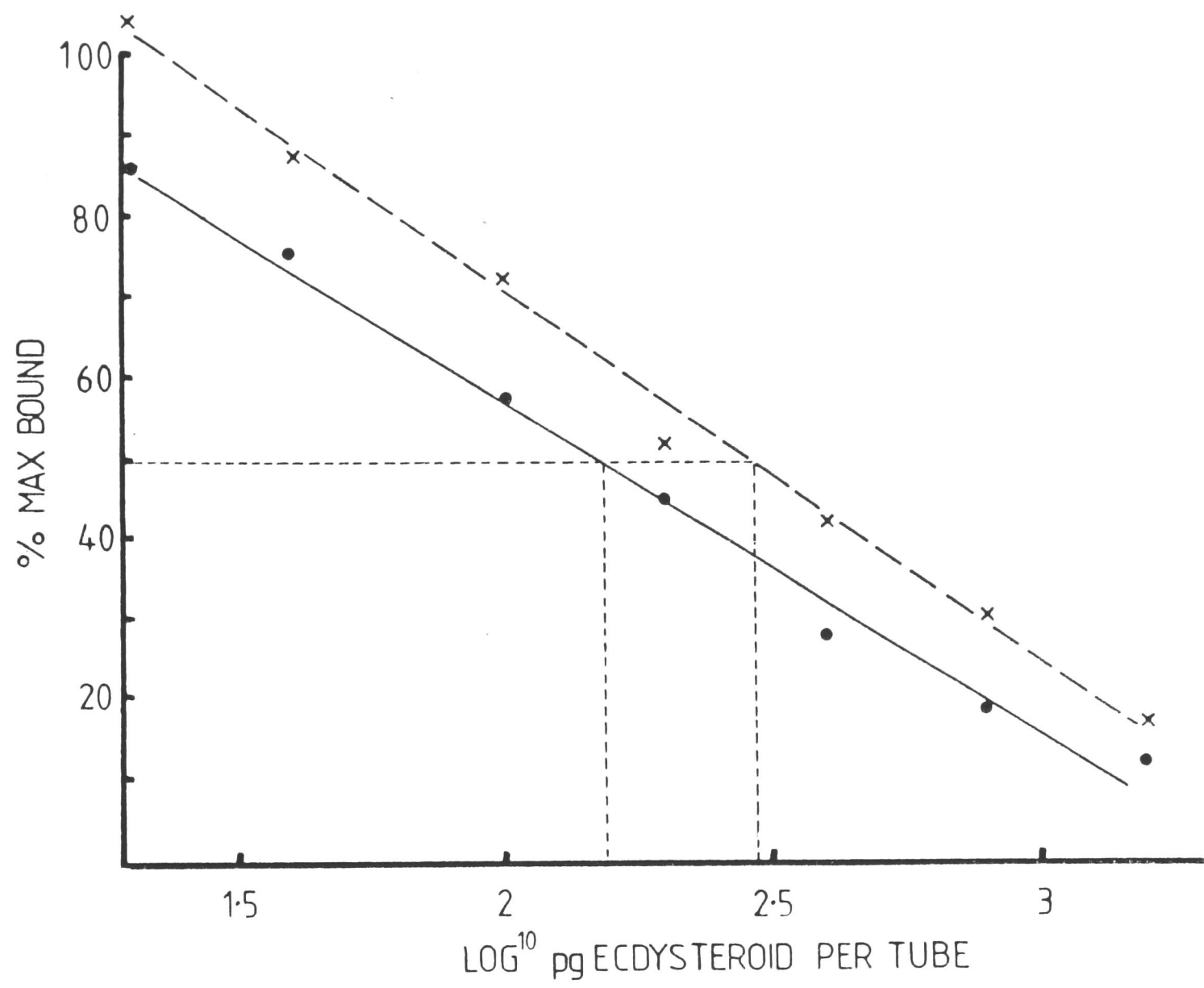


Figure A-3: The standard curves for ecdysone (●—●) and 20-hydroxyecdysone (X--X) in the Horn-22 radioimmunoassay, plotted as the percentage of the maximum bound count against the logarithm of the ecdysteroid concentration. 100 $\mu$ l of 0.05% Horn-22 and 100 $\mu$ l of radioactive ecdysone (18000 dpm) were added to each tube. Ecdysteroid concentrations were 20, 40, 100, 200, 400, 800, and 1600 pg/tube. Assay termination by second antibody precipitation, 100 $\mu$ l of 0.75% donkey anti-rabbit IgG per tube. Methods as described in the text. The 50% maximum binding points were used to calculate the ratio of ecdysone to 20- hydroxyecdysone binding (1.92). Each point represents the mean of 3 determinations. Ecdysone, slope = -39.3, correlation = 0.989; 20- hydroxyecdysone, slope = -44.9, correlation = 0.997.

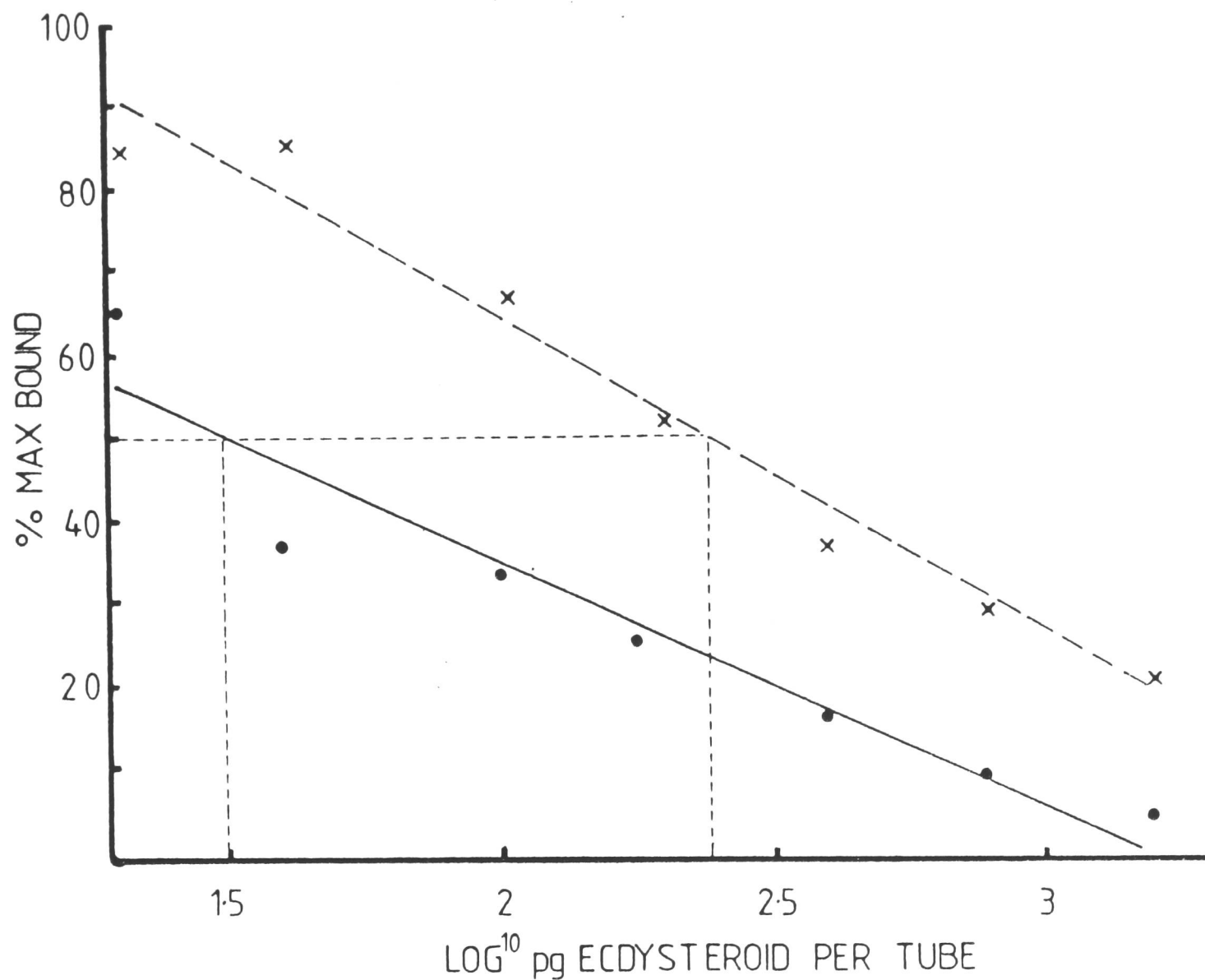


Figure A-4: The standard curves for ecdysone (●—●) and 20-hydroxyecdysone (X--X) in the Horn-2 radioimmunoassay plotted as the percentage of the maximum bound count against the logarithm of the ecdysteroid concentration. 100  $\mu$ l of 0.04% Horn-2, 100  $\mu$ l of radioactive ecdysone (18000 dpm) and 100  $\mu$ l of 0.5% donkey anti-rabbit IgG were added to each tube. Other assay conditions as for figure A-3. Ecdysone:20-hydroxyecdysone binding ratio = 7.90. Each point represents the mean of 3 determinations. Ecdysone, slope = -28.1, correlation = 0.961; 20-hydroxyecdysone, slope = -36.4, correlation = 0.987.

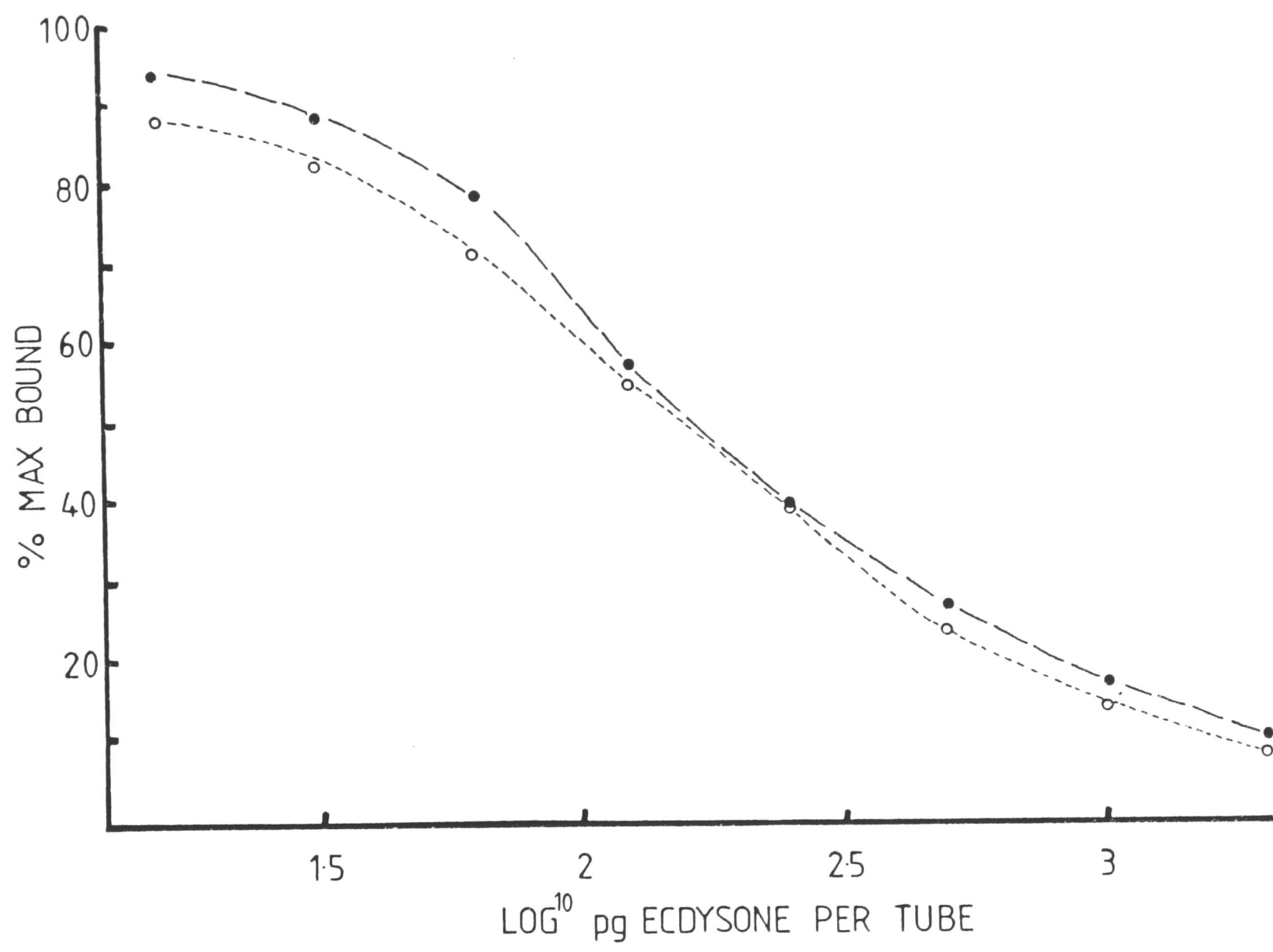


Figure A-5: The effect of using either second antibody precipitation ● (conditions as for figures A-3 and A-4) or protein-A assay termination ○ methods on the ecdysone standard curve produced with Horn-22 antiserum. Ecdysone concentration was 15, 31, 62, 125, 250, 500, 1000, and 2000 pg/tube. 20ul of either a 5% wet wt./vol. protein-A suspension or 20μl of 2.5% donkey anti-rabbit IgG added to each appropriate tube.



The conditions of radioimmunoassay used in the course of the work reported in this thesis were different in the various sections. The specific conditions used will now be outlined.

Chapter 3: Haemolymph ecdysteroid analysis.

100µl of 0.05% Horn-22 antiserum

100µl radioactive ecdysone (18000 dpm)

Ecdysone standards, 20,40,100,200,400,800,1600 pg per tube

100µl of 0.75% Donkey anti-rabbit second body

Samples, 20–25µl of 1:9 haemolymph:methanol

Chapter 3: Specific ecdysteroid analysis

100µl of 0.05% Horn-22, or 0.04% Horn-2 antiserum

100µl radioactive ecdysone (18000 dpm)

Ecdysone standards, 15,31,62,125,250,500,1000,2000 pg per tube

20µl 5% wet wt./vol protein-A suspension

Chapter 4: *In vitro* prothoracic gland ecdysteroid production

Conditions as for chapter 3, haemolymph analysis except for,

Ecdysone standards, 15,31,62,125,250,500,1000,2000 pg per tube

## II. Appendix 2. Papers published from this thesis

Richard,D.S., Saunders,D.S., Egan,V.M. and Thomson,R.C.K. (1986) The timing of larval wandering and puparium formation in the flesh-fly *Sarcophaga argyrostoma* *Physiol. Ent* **11**, 53-60.

Richard,D.S. and Saunders,D.S. (1987a) Prothoracic gland function in diapause and non-diapause destined larvae and pupae of *Sarcophaga argyrostoma* and *Calliphora vicina* *J. Insect Physiol.* In Press.

Richard,D.S. and Saunders,D.S. (1987b) *In vitro* activation of prothoracic glands from diapause and non-diapause destined *Sarcophaga argyrostoma* and *Calliphora vicina*. *Proc. International conference on insect neurochemistry and neurophysiology*. In Press.

Richard,D.S. Warren,J.T.,Saunders,D.S. and Gilbert,L.I.(1987) Haemolymph ecdysteroid titres in diapause and non-diapause destined larvae and pupae of *Sarcophaga argyrostoma* *J. Insect Physiol* **33**, 115-122

## The timing of larval wandering and puparium formation in the flesh-fly *Sarcophaga argyrostoma*

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**ABSTRACT.** In mixed-age cultures of the flesh-fly, *Sarcophaga argyrostoma* (Robineau-Desvoidy), the initiation of larval wandering (exodus behaviour) occurs as a gated circadian rhythm. In light–dark (LD) cycles, most of this activity occurs in the dark, except in very short nights, or in certain phase relationships between the rhythm and light cycle. When transferred from series of LD cycles into continuous darkness (DD), cultures show a weakly persistent free-running rhythm with a period of about 21 h. However, after transfer of first instar larvae from continuous light (LL) to DD, no such rhythm is observed. In contrast to larval exodus, formation of the puparia occurs at any stage of the LD cycle. The physiological mechanisms underlying this gated exodus behaviour, and its possible selective advantages, are discussed.

**Key words.** *Sarcophaga argyrostoma*, larval wandering, gating, circadian rhythm.

### Introduction

Physiological and behavioural events in the life cycles of insects may be regulated by biological clocks (Saunders, 1982). Pittendrigh (1954) showed that pupal eclosion in mixed-age populations of the fruit fly *Drosophila pseudoobscura* occurs as a circadian rhythm. In light–dark cycles, eclosion occurs during an allowed zone or ‘gate’ close to dawn, when conditions are favourable for adult survival. Other events may not be coupled to the circadian system, presumably when such behaviour offers no selective advantage to the species concerned (Pittendrigh, 1958).

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In the flesh-fly *Sarcophaga argyrostoma* (Robineau-Desvoidy), pupal eclosion is under the control of a gated circadian rhythm similar to that noted above (Saunders, 1976). The induction of pupal diapause (Saunders, 1971; Denlinger, 1972) and the length of larval development (Saunders, 1972) have been shown to be strongly coupled to the photoperiodic clock. The present paper examines the onset of larval wandering behaviour (exodus from the food) and subsequent puparium formation for evidence of clock control.

### Materials and Methods

Stock cultures of *S. argyrostoma* were raised as adults in cages at 25°C and continuous light. The

flies were supplied with sugar and water *ad libitum*, and with a daily supply of fresh beef muscle. Newly deposited larvae were transferred on pieces of meat to a supplementary diet made from yeast, dried milk, agar and water cast in plastic dishes. Fully fed larvae were allowed to wander into dry sawdust to form puparia.

In the experimental cultures, 200–300 first instar larvae deposited over a period of 2 h or 24 h were set up as above but exposed to a variety of light–dark (LD) cycles, or to continuous light (LL) or dark (DD) in light-tight boxes at  $20 \pm 0.5^\circ\text{C}$ . Each box was fitted with a 4 W Philips striplight regulated by a Venner timeswitch. Temperatures in the boxes and within the cultures were measured by a Rustrak temperature probe. The lights-on portion of the LD cycle did not cause a significant rise in temperature but temperatures within the cultures rose  $2\text{--}3^\circ\text{C}$  above ambient before the onset of exodus.

When wandering commenced, larvae falling into the sawdust were collected and counted hourly until all of them had left the food. Those larvae leaving the food during the dark phase of the cycle were counted under a red safe light. The timing of larval wandering behaviour was in all cases non-random, usually occurring in a number of discrete peaks in both LD and DD regimes. Following Winfree (1970) in his study of pupal eclosion in *Drosophila pseudoobscura*, peaks of 'activity' were defined as the densest 8 h in each cycle. The distribution of larval exodus within and around each peak thus defined was frequently skewed.

In the experiments on puparium formation, newly wandering larvae were placed individually in  $6 \times 3.5$  cm plastic tubes containing 1 ml of dry sawdust to avoid the complicating effects of crowding on the rate of pupariation (Bradley, 1984). Every hour, newly-formed (white or pale brown) puparia were recorded: the time of pupariation could then be related to both local time and to the time of wandering. A control group of larvae was manually extracted from the food before exodus began and then examined for periodicity of pupariation.

## Results

### *The timing of larval exodus in daily light cycles*

Seven cultures of larvae, deposited during a 2 h period by flies in LL, were set up in LD

12:12 h,  $20 \pm 0.5^\circ\text{C}$  with the light–dark transition occurring 4 h later in each regime (Fig. 1). Larval wandering commenced after 5 days and continued for about 36 h with all cultures showing one or two discrete peaks of exodus. For the cultures A, C, D and E, the peaks occurred close to the onset of darkness, the peaks following the sequentially later transitions from light to dark. In cultures B, F and G, some larvae left the food during the light phase, suggesting that they were able to follow a delay in lights-off only to a certain point before phase-jumping back to an earlier transition.

Fig. 1H shows the same data summed according to photoperiod. This treatment produced an artificial or 'synthetic' mixed-age population (Pittendrigh, 1966) and clearly shows that larval exodus is rhythmic in an LD 12:12 h light cycle.

### *Larval exodus in light cycles with varying night lengths*

Seven cultures were established in differing light–dark cycles with the lights-on transition occurring at the same local time (Fig. 2, B–H). One culture was set up in DD (Fig. 2A) and one in LL (Fig. 2I). The larvae were deposited over a 24 h period to provide a mixed-age population resulting in a greater spread of developmental age, and hence an increased number of activity peaks.

Most cultures produced larval exodus patterns with two or three clear peaks. In the longer night lengths (Fig. 2, B and C) larval exodus occurred exclusively in the dark, but as night length shortened, and the LD transition occurred later relative to larval age (Fig. 2, D and E), the peaks showed a stronger association with the onset of darkness. Culture F showed a peak of wandering in the light as the larvae phase-jumped back to an earlier dark phase (Fig. 2G). In the shortest night (Fig. 2H), exodus commenced in the dark but continued into the next light phase. Wandering behaviour in DD (Fig. 2A) following a transfer from LL after larviposition, and in LL (Fig. 2I) was arrhythmic.

### *Transfer from LD to continuous darkness*

To investigate the possible circadian basis of the rhythmic initiation of wandering, mixed-age cultures of larvae were transferred to DD after two or three cycles of LD 12:12 h with the final

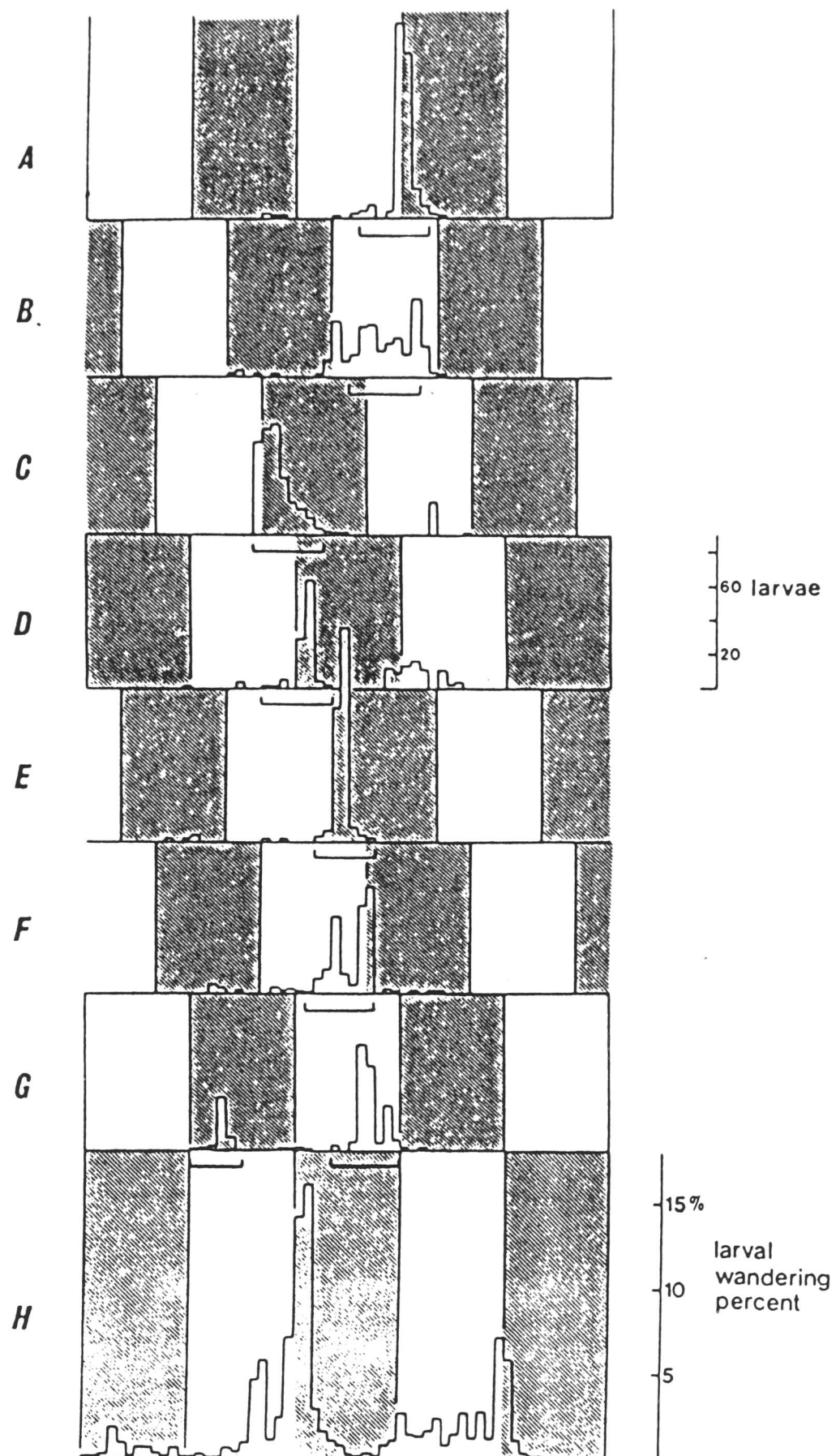


FIG. 1. Larval wandering (exodus behaviour) in seven cultures (A-G) of the flesh-fly *Sarcophaga argyrostoma*, maintained in LD 12:12 h,  $20 \pm 0.5^\circ\text{C}$ , , with different phase relationships between the light cycle and larval age. The horizontal brackets mark the most dense 8 h of activity in each recognized peak. H, data from A-G summed according to the light-dark cycle, giving a 'synthetic' mixed-age population.

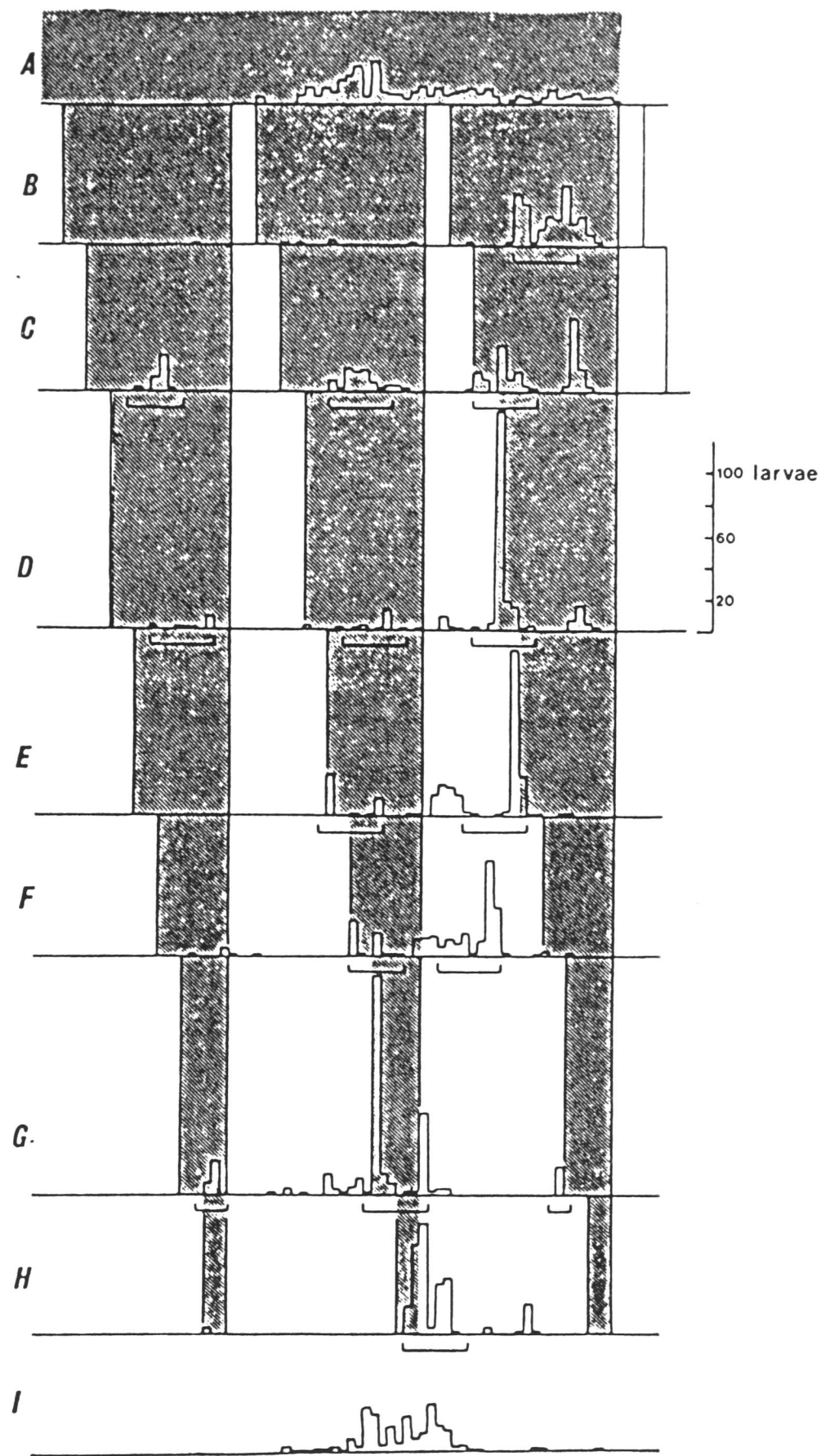


FIG. 2. Larval wandering behaviour in nine cultures (A–I) exposed to light cycles of DD; LD 3:21, 6:18, 9:15, 12:12, 15:9, 18:6 and 21:3 h; and LL respectively (with the lights-on transitions occurring at the same local time). Horizontal brackets mark the most dense 8 h of activity in each peak.

transfer occurring at different local times (Fig. 3, A–G). Results shown in Figs. 1 and 2 have already demonstrated the association of wandering with a continuous LD regime.

Cultures A, B, D and E showed two or three discrete peaks, clearly 'free-running' in DD with a circadian period of less than 24 h. Cultures C, F and G, however, showed a breakdown in this pattern possibly as a result of a transient state (C) or the inability of the rhythm to persist in those cultures which had spent the longest time in darkness (F and G). Since Fig. 2A showed that a simple transfer from LL to DD was insufficient to initiate the circadian rhythm, it is understood that exposure to several LD cycles is required for the entrainment of the system, and that the rhythm, once initiated, does not persist for long in the absence of further time cues.

The circadian period ( $\tau$ ) of the rhythm in DD in the clearer 'free-runs' of Fig. 3 (A, B, D and E) was about 21 h.

#### *The timing of puparium formation*

Two mixed-age cultures of larvae were set up in the LD 12:12 h. In one (Fig. 4A) the fully fed larvae were extracted manually from their food just before exodus commenced; in the other (Fig. 4B) larvae were allowed to leave their food naturally. In both cultures, larvae were transferred to single tubes and puparium formation was recorded every hour.

Although larval exodus was clearly gated (Fig. 4b) and largely nocturnal, puparium formation in both groups (A and B) was at random with no evidence of rhythmicity. Fig. 4C also shows pupariation in B arranged according to the time, in hours, from larval exodus, rather than according to larval age or the LD cycle; once again, there was no evidence of rhythmicity. The range of pupariation times in Fig. 4B (about 84 h) was expected since the spread of larval exodus was about 60 h. However, the enormous range of pupariation times (49–133 h) observed when the data are plotted against time since exodus (Fig. 4C), was surprising. The result illustrates that little, if any, temporal control operates for puparium formation, in marked contrast to the stronger circadian regulation of larval exodus.

#### **Discussion**

These results show that the onset of wandering behaviour in fully fed larvae of *Sarcophaga*

*argyrostoma* occurs largely at night or shortly after lights-off. In a population therefore, exodus is rhythmic with a series of nocturnal peaks of activity similar to those described for blowflies (Smith *et al.* 1981; Spradbery *et al.*, 1983; Roberts, 1984). In *S. argyrostoma* this rhythm is partly governed by the circadian system so that it free-runs in continuous darkness with an endogenous period close to 21 h. Since each larva leaves its food only once, a mixed-age population is required to demonstrate such a rhythm; the rhythm is therefore 'gated' as in pupal eclosion (Pittendrigh, 1954; Saunders, 1976). In marked contrast, pupariation in *S. argyrostoma* is evidently not gated and can occur at any time of the night or day.

When compared with pupal eclosion (Saunders, 1976), it is clear that the circadian regulation of larval exodus is rather weak, with some of its peaks being poorly defined, and an ability to free-run in darkness being restricted to those cultures recently exposed to several LD cycles rather than to simple 'step-down' from light to dark. This may reflect either a damping of the oscillatory system with time or a developing asynchrony between the rhythms of individual larvae.

Working with *S. bullata*, Roberts (1984) demonstrated that the nocturnal peak of larval wandering coincided with a pulse of ecdysteroids in the haemolymph, itself immediately preceded by a heightened release of ecdysone from the ring gland (as measured *in vitro*). This was presumably the result of an even earlier release of prothoracicotropic hormone (PTTH) from the brain. It is this release of PTTH that is thought to be the primary gated event. In the tobacco hornworm *Manduca sexta*, it has been shown that the pulsatile release of PTTH prior to larval exodus is a gated event, but that before pupariation is not (Truman & Riddiford, 1974; Gilbert *et al.*, 1981; Dominick & Truman, 1984). Although the neuroendocrine events governing gated mechanisms are largely unknown, it is probable that the cerebral circadian clock issues a daily neural signal which leads to PTTH release only when other factors permit. Since a high titre of juvenile hormone is known to inhibit PTTH release in final instar *M. sexta* larvae (Nijhout & Williams, 1975), a drop in the titre of this hormone may be the permissive factor involved.

It is interesting to compare developmental events in *S. argyrostoma* which are known to be

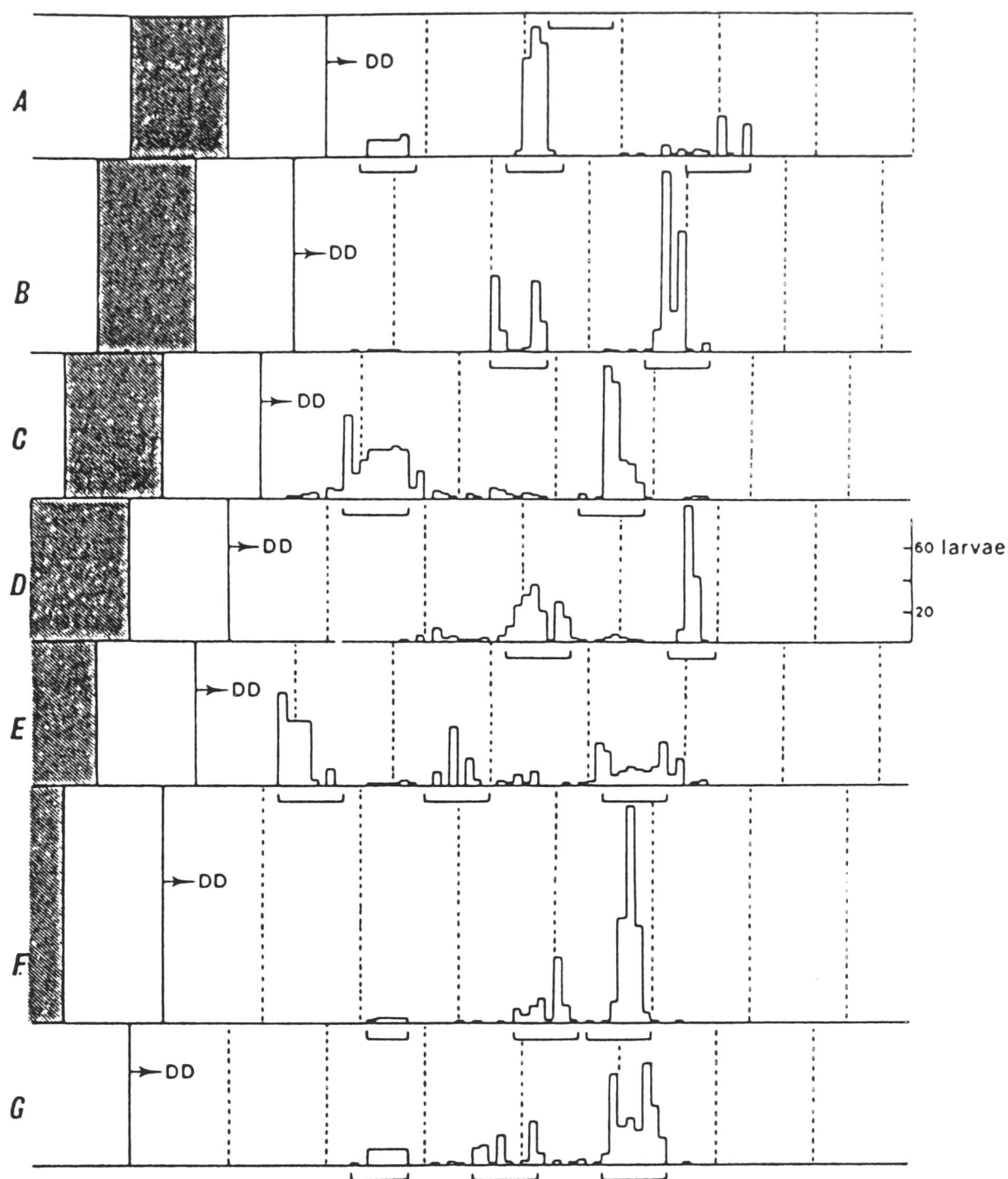


FIG. 3. Larval wandering behaviour in seven cultures (A–G) released from three cycles of LD 12:12 h into continuous darkness (DD) at different local times. Horizontal brackets mark the most dense 8 h of activity in each peak. Vertical dotted lines mark 12 h intervals from the onset of DD.

clock controlled (larval exodus and pupal eclosion, for example) with those which are not (puparium formation), and to consider the possible selective advantages for such control. Clearly there can be little advantage gained by forming the puparium at a certain phase of the day–night cycle since the larva is already subterranean at this point. Pupal eclosion and larval wandering, on the other hand, are both gated, but differ in both phase and circadian period, the former occurring close to dawn and showing a

free-running period close to 24 h (Saunders, 1976), and the latter occurring close to dusk with a period of about 21 h. The selective advantage of flies emerging at dawn might be that wing expansion is more effectively accomplished at high humidity (Pittendrigh, 1958), or merely that a mass dawn emergence synchronizes a population of diurnally-active flies for the day ahead. For eclosion, a circadian period of close to 24 h enables the developing adult flies, lying underground for several weeks, to emerge at the



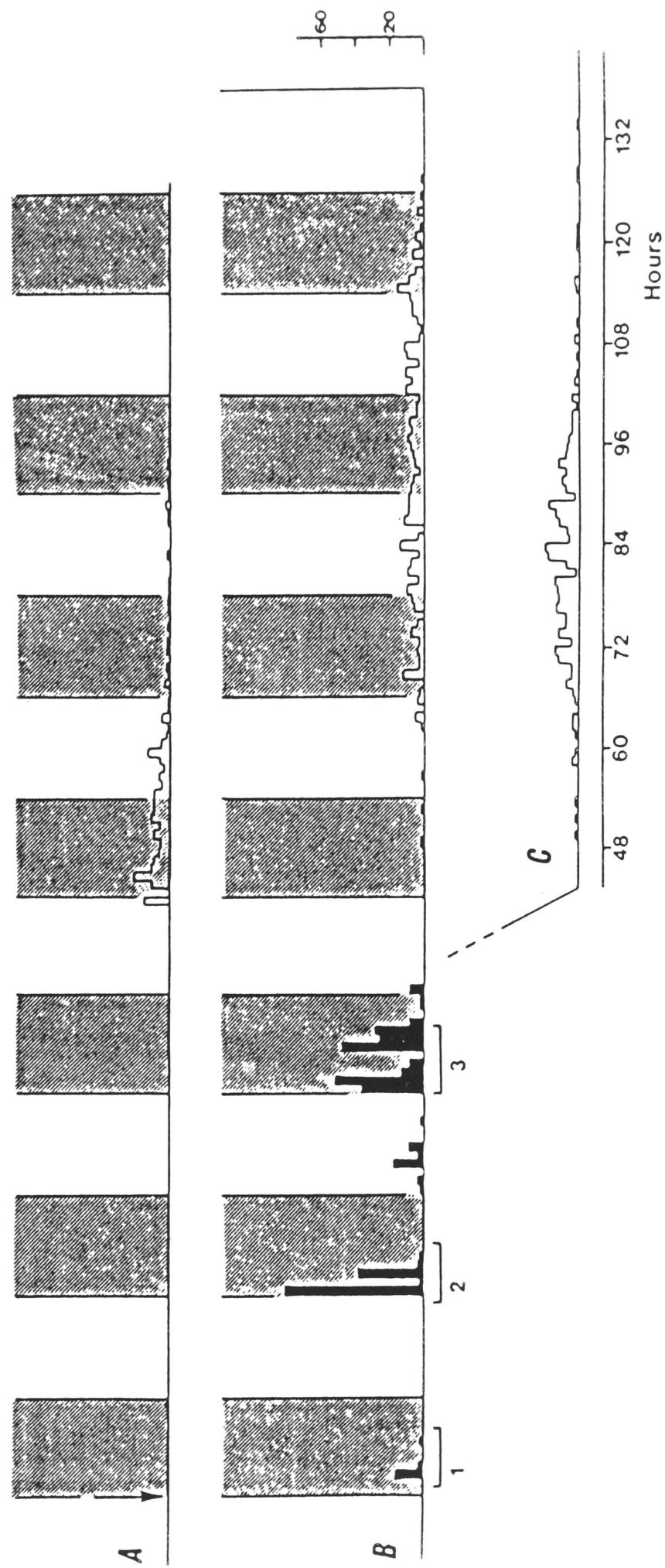


FIG. 4. (A) Distribution of puparium-formation in a mixed-age culture of *S. argyrostoma* whose fully fed larvae were manually extracted from their food (at arrow). (B) Larval wandering (solid histograms) and puparium formation (open histograms) in a culture allowed to wander naturally. (C) Distribution of puparium-formation in culture B plotted in hours from larval wandering. Horizontal brackets in B mark the most dense 8 h of activity in each peak.

most appropriate time, measured in multiples of 24 h since the last-seen dawn.

The advantages of dusk or nocturnal wandering of the larvae are more problematical. Roberts (1984) argued that such behaviour enables the larvae to avoid diurnal predators, those larvae awaiting the next gate within the carcass enjoying a safe refuge. On the other hand, nocturnal wandering might expose the larvae to another set of predators, and staying within the carcass during the day merely provide a convenient focus for carrion-feeding birds. The selective advantage of nocturnal wandering, therefore, may lie in the avoidance of undue desiccation.

The clock governing pupal eclosion is phase-set by light during embryonic and early larval development, and runs with a period close to 24 h (Saunders, 1976). The clock governing larval wandering is probably also entrained during the same early developmental stages, but then shows a much shorter period (about 21 h). The two systems therefore apparently run concurrently but possess different properties. This raises the possibility that they are regulated by two separate circadian oscillators, rather like those described for pupal eclosion and adult locomotor activity in *Drosophila pseudoobscura* (Engelmann & Mack, 1978).

### Acknowledgments

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## PROTHORACIC GLAND FUNCTION IN DIAPAUSE AND NON-DIAPAUSE *SARCOPHAGA ARGYROSTOMA* AND *CALLIPHORA VICINA*

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**Abstract**—The *in vitro* activation of isolated prothoracic glands from *Sarcophaga argyrostoma* (Robineau-Desvoidy) was accomplished with extracts of prepupal brains. The action of prothoracicotrophic hormone (PTTH) could be mimicked by the use of cAMP analogues and a phosphodiesterase inhibitor, suggesting the involvement of a cyclic nucleotide-mediated secondary messenger system. This was further supported by the removal of calcium ions thereby lowering the effect of the PTTH extracts *in vitro*. Cyclic GMP and its derivatives had no effect on ecdysone synthesis. The PTTH extract from *Sarcophaga* was found to be equally effective at stimulating the production of ecdysone by isolated *Calliphora vicina* (Meigen) ring glands. The nature and time course of ring gland competency in diapause in both *Sarcophaga* and *Calliphora* was investigated. As the *Sarcophaga* population entered pupal diapause at 96 h after pupariation at 18°C, the prothoracic glands became refractory to PTTH, taking 24 h to lose their competency to respond to this hormone. The levels of ecdysone produced were below the limits of detection of the radioimmunoassay used. The glands could not be stimulated by 10 mM cyclic nucleotides indicating that the block of PTTH action occurred beyond the proposed stage of cyclic nucleotide mediation. The *Calliphora* population, which has a larval diapause, took 6 days to reach this refractory state. Their prothoracic glands continued to produce ecdysone at the basal rate throughout diapause and this basal rate could not be altered by the application of PTTH or cyclic nucleotides. Following a temperature increase from 11 to 25°C, all diapausing *Calliphora* had regained prothoracic gland competency within 24 h and had pupariated within 36 h. When isolated brain-ring gland complexes from diapausing *Calliphora* were incubated at 25°C, no recovery of ring gland competency was noted, indicating the requirement for *in vivo* reactivation after diapause. The levels of PTTH in pre-diapause and day-35 post-pupariation diapause brains of *Sarcophaga* were shown to be similar to those of non-diapause destined prepupal brains.

**Key Word Index:** Prothoracicotrophic hormone, ecdysone, diapause, cyclic nucleotides, *Calliphora vicina*, *Sarcophaga argyrostoma*

### INTRODUCTION

Many insects enter a diapause state, induced by changing photoperiod, to avoid times of environmental stress. The flesh-fly *Sarcophaga argyrostoma* (Robineau-Desvoidy) which enters pupal diapause in winter (Fraenkel and Hsiao, 1968), exhibits a temperature-modified photoperiodic response such that long days promote continuous development, whereas short days coupled with lower temperatures induce diapause (Saunders, 1981). Denlinger (1971) showed that the embryonic and early larval stages of *Sarcophaga* spp. are the most sensitive to photoperiod, although diapause does not occur until the pupal stage. *Calliphora vicina* (Meigen) displays a larval diapause induced principally by short days experienced by the female parent (Vinogradova and Zinovjeva, 1972; Saunders *et al.*, 1986). In the diapause state, both species have a very low haemolymph ecdysteroid titre (Richard *et al.*, 1986; this paper), presumably as a result of the regulatory effects of the neuroendocrine system, which interrupt the release of prothoracicotrophic

hormone (PTTH) from the brain-retrocerebral complex (Bowen *et al.*, 1984a, b).

Ecdysone is primarily produced by the prothoracic glands in most insect species. In the Lepidoptera, two of these glands exist separately from any other endocrine organs. The Diptera however, have a fused retrocerebral complex, the ring gland, which contains the prothoracic glands, as well as the corpus cardiacum and the corpus allatum. Bollenbacher *et al.* (1976) established that isolated ring glands from *Sarcophaga bullata* synthesized ecdysone rather than any other ecdysteroid. Although Redfern (1984) found that *Drosophila* ring glands sometimes synthesized 20-deoxymakisterone A under certain dietary conditions, it is assumed that the major steroid produced by the prothoracic gland is ecdysone itself. In 1979, Bollenbacher *et al.* developed an *in vitro* assay for PTTH action on isolated prothoracic glands from the tobacco hornworm *Manduca sexta*. This assay involved the incubation of the glands in partially purified *Manduca* brain extracts. The incubation medium was then assayed for ecdysone by radioimmunoassay. Roberts *et al.* (1984) adapted this *in vitro* assay for PTTH analysis in *Sarcophaga bullata* using isolated ring glands.

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In addition to the reduction in PTTH release that is probably the primary cause of diapause induction, Bowen *et al.* (1984a) found that the prothoracic glands become refractory to PTTH stimulation, thereby further lowering the level of circulating ecdysone. In non-diapause *Manduca*, PTTH acts on the prothoracic gland via a calcium ion-sensitive cyclic adenosine monophosphate (cAMP) secondary messenger-mediated system (Smith *et al.*, 1984, 1985). In diapausing hornworm pupae however, the prothoracic gland is not competent to respond to cyclic nucleotides, or to agents that work by raising the intracellular levels of these nucleotides, for example, forskolin, an adenylate cyclase stimulator, or 1-methyl-3-isobutylxanthine (MIX), a phosphodiesterase inhibitor (Smith *et al.*, 1986).

In this study, the ability of ring glands from *Sarcophaga argyrostoma* and *Calliphora vicina* to respond to brain extracts from prepupal *Sarcophaga* by producing and releasing RIA positive material is investigated. A role for cyclic nucleotides and calcium in prothoracic gland function in diapausing and non-diapausing individuals of these two flies is also investigated. Changes in ring gland function as the insects enter diapause are determined in both species, and the emergence from diapause is followed in *Calliphora*. Data regarding the PTTH content of pre-diapause and deep diapause *Sarcophaga* brains are also presented.

#### MATERIALS AND METHODS

##### Culture maintenance

Stock cultures of *Sarcophaga* and *Calliphora* were raised in cages at 25°C under continuous light as described in Richard *et al.* (1986). Diapause-destined *Sarcophaga* were raised by maintaining the adults at 25°C, and then transferring the larvae to 18°C, both stages under 12 h light, 12 h dark. Diapause larvae of *Calliphora* were produced by keeping the adults at 20°C and 12 h light, 12 h dark. Eggs deposited on day 12 were then raised at 11°C in constant darkness (Saunders *et al.*, 1986).

##### Ecdysteroid radioimmunoassay

Radioimmunoassay termination was accomplished by donkey anti-rabbit immunoglobulin-G second antibody (Scottish Antibody Production Unit) against Horn-22 antisera (kindly supplied by Professor L. I. Gilbert, University of North Carolina, Chapel Hill, NC 27514, U.S.A.) against an ecdysone standard curve as reported in Richard *et al.* (1986). All results are expressed as ecdysone equivalents. None of the chemicals used in these investigations for ring gland incubation were found to interfere with the RIA.

##### In vitro assay

The *in vitro* ring gland assay was conducted in a 10 µl hanging drop suspended over Grace's medium (Gibco) in an upturned Petri dish to avoid undue evaporation, according to the method of Roberts *et al.* (1984). All incubation solutions were made up with Grace's medium containing an additional 10 mM CaCl<sub>2</sub>, with the exception of the EDTA experiment when this was omitted. Ring glands were incubated at 25°C in darkness. After 4 h, the ring

glands were removed from the incubation mixtures and two 5 µl aliquots assayed directly for ecdysone by RIA.

Brain extracts, presumably containing PTTH, were made according to the method reported by Roberts *et al.* (1984) using 4 h *Sarcophaga* prepupal brains for the standard extract. This extract was used for both the *Sarcophaga* and *Calliphora* incubation studies. Other extracts were prepared as described in the relevant sections. Cyclic nucleotides, EDTA, and MIX (1-methyl-3-isobutylxanthine) were purchased from the SIGMA Chemical Co. The concentrations of reagents used in these investigations were based on those used by Smith *et al.* (1984, 1985).

#### RESULTS

##### In vitro activation of the ring gland by prothoracicotropic hormone

The activation of isolated *Sarcophaga* post feeding larval ring glands by prepupal (Fraenkel and Bhaskaran, 1973) brain extracts, assumed to contain PTTH, is shown in Fig. 1. This work followed the protocol proposed by Roberts *et al.* (1984). The glands did not respond to PTTH stimulation until more than 0.25 brain equivalents were administered. At this concentration, the amount of ecdysone produced increased from the basal level of less than 500 pg/4 h to over 1300 pg/4 h, an activation ratio approaching 3. No increase in ecdysone production was noted when the glands were incubated with a control extract from one ventral ganglion. Ring glands removed from red-spiracular larvae of *Sarcophaga* were more active and more responsive to PTTH stimulation than those from post-feeding larvae. A marginally higher basal level of ecdysone synthesis ( $620 \pm 80$  pg/4 h) increased steadily with increasing PTTH concentration to a maximum of over 2100 pg/4 h. Ecdysone production maxima were reached in both dose-response

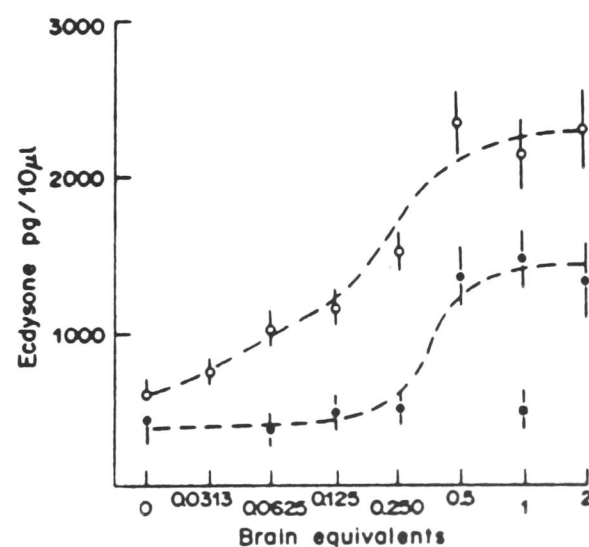


Fig. 1. Dose-response curves for the *in vitro* activation of *Sarcophaga* post-feeding larval (●---●) and redspiracular larval (○---○) ring glands incubated with PTTH extracts from 4 h prepupal brains in Grace's medium plus 10 mM CaCl<sub>2</sub> for 4 h at 25°C. An extract of ventral ganglion was used as a control (■) with post-feeding larval ring glands. Each point represents the mean  $\pm$  SEM of 5 to 10 separate activation analyses. Results expressed as ecdysone equivalents.

curves when the PTTH concentration reached 0.5 brain equivalents.

*The effects of cyclic nucleotides on isolated ring glands in vitro*

Following the dose-response experiments, a 0.5 brain equivalent measure of 4 h prepupal *Sarcophaga* brain extract was adopted as the standard PTTH dose. In each experiment, a blank was included which consisted of a group of ring glands ( $n = 5$ ) from the appropriate developmental stage, incubated in Grace's medium to determine the basal level of ecdysone production. Figure 2 shows that the inclusion of 0.1 mM 1-methyl-3-isobutylxanthine (MIX), a potent phosphodiesterase inhibitor which increases intracellular cyclic nucleotide levels (Ravankar and Robins, 1982), in the incubation medium increased the level of ecdysone production from both the basal and PTTH stimulated levels in *Sarcophaga*. The removal of calcium ions by the inclusion of 10 mM EDTA negated the effect of PTTH thereby lowering the amount of ecdysone produced to basal levels.

The involvement of cyclic nucleotides in the mediation of PTTH action in *Sarcophaga* is more fully investigated in Fig. 3. The cyclic adenosine monophosphate (cAMP) analogues dibutyryl cAMP and 8-bromo cAMP (10 mM) were investigated for their effects on ecdysone production. Dibutyryl cAMP which enters cells more rapidly than cAMP (Ravankar and Robins, 1982) was associated with a very large increase in ecdysone synthesis, whereas

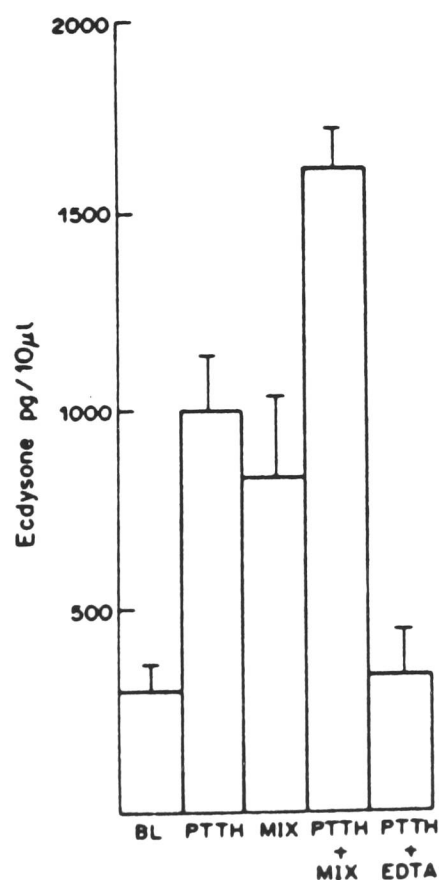


Fig. 2. The effects of the phosphodiesterase inhibitor, 1-methyl-3-isobutylxanthine (MIX, 0.1 mM) and the calcium chelator EDTA (10 mM) on the action of PTTH (0.5 brain equivalents) on *Sarcophaga* post-feeding larval ring glands *in vitro*. BL represents the basal level of ecdysone synthesis by these glands. Each point represents the mean  $\pm$  SEM of 5–10 separate activation analyses. Results expressed as ecdysone equivalents.

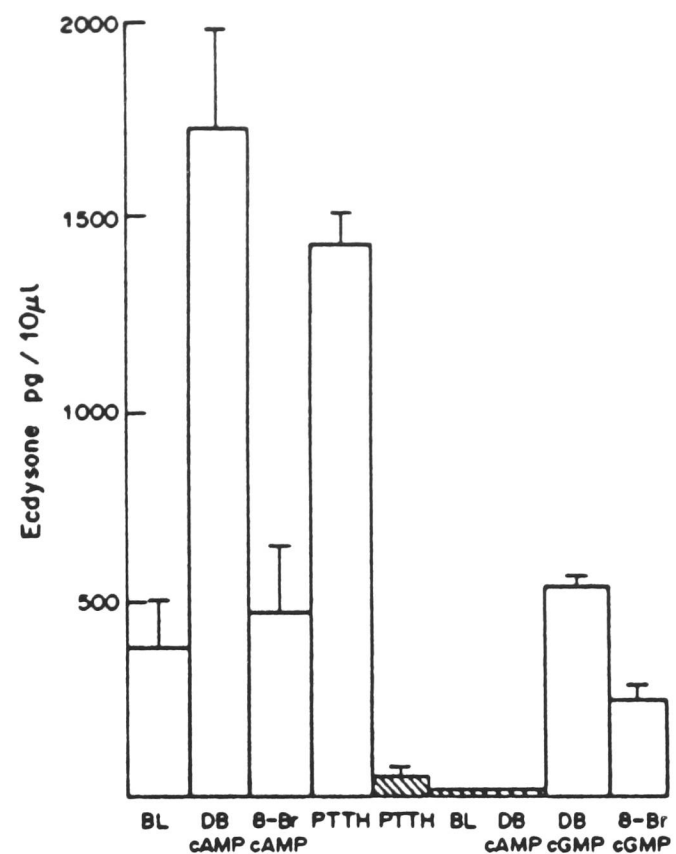


Fig. 3. The effect of 10 mM cyclic nucleotides: dibutyryl cAMP (DBcAMP), 8-bromo cAMP (8-BrcAMP), dibutyryl cGMP (DBcGMP) and 8-bromo cGMP (8-BrcGMP); and PTTH (0.5 brain equivalents) on *Sarcophaga* post-feeding larval ring glands incubated *in vitro* for 4 h at 25°C (open bars) and on pupal diapause ring glands (Day 28 after pupariation) (hatched bars). BL represents the basal levels of ecdysone synthesis by the appropriate ring glands. Each bar represents the mean  $\pm$  SEM of 6–10 separate activation analyses. Results expressed as ecdysone equivalents.

8-bromo cAMP which is more resistant to degradation than cAMP was not. The cyclic guanosine monophosphate (cGMP) analogues, dibutyryl cGMP and 8-bromo cGMP had little effect on the basal levels of ecdysone production.

Figure 4 shows data obtained from *Calliphora* ring glands exposed to the standard *Sarcophaga* brain extract. The large increase from basal levels to 1500 pg/4 h demonstrates a degree of similarity in response to PTTH and possibly therefore in PTTH structure and function. Similar increases in ecdysone production to those in *Sarcophaga* were associated with cyclic nucleotide stimulation.

*The competency of ring gland function in diapause*

The cross-hatched bars of Figs 3 and 4 refer to ring glands removed from day-28 post-pupariation diapausing *Sarcophaga* pupae (Fig. 3) and day-32 post-oviposition diapausing *Calliphora* larvae (Fig. 4). The basal level of ecdysone production by *Sarcophaga* is below the limit of detection of the radioimmunoassay used (30 pg). This basal level did not rise even when PTTH was added to the medium. No stimulation of ecdysone production was noted with 10 mM dibutyryl cAMP either, indicating that a block to ring gland function existed further downstream from the cyclic nucleotide link.

Figure 4 shows similar data obtained from diapausing *Calliphora* ring glands. Whilst there is no increase in ecdysone production associated with



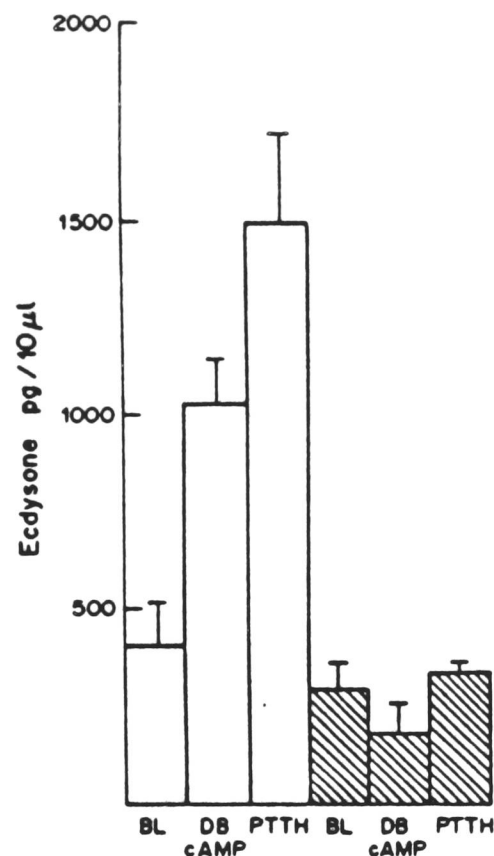


Fig. 4. The effect of 10 mM dibutyryl cAMP (DBcAMP) and PTTH (0.5 brain equivalents from *Sarcophaga* prepupal brains) on ring glands from non-diapause post-feeding (open bars) and Day-32 post-oviposition diapause (hatched bars) *Calliphora* larvae. BL represents the appropriate basal level of ecdysone synthesis. Each bar represents the mean  $\pm$  SEM of 5–10 separate activation analyses. Results expressed as ecdysone equivalents.

PTTH or dibutyryl cAMP treatment, the basal levels are much higher than those for *Sarcophaga*. The diapause basal level is the same as the non-diapause basal level (about 300–400 pg/ring gland/4 h).

#### Time-course of ring gland deactivation in diapause

Larvae of *Sarcophaga* were raised under diapause-inducing conditions and newly formed puparia collected. Groups of ring glands ( $n = 5–10$ ) were removed daily and incubated in the standard 0.5

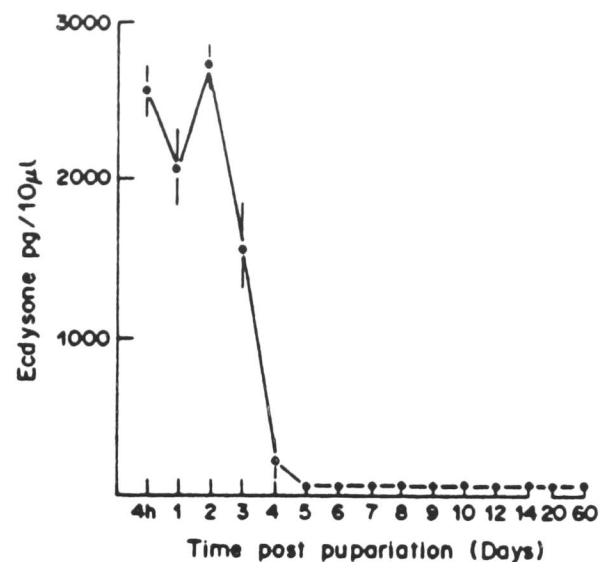


Fig. 5. The competency of ring glands taken from post-pupariation diapause-destined *Sarcophaga* (maintained under LD12:12 at 18°C) to synthesise ecdysone in response to incubation with 0.5 brain equivalents of 4 h *Sarcophaga* prepupal brain extract. As the pupae enter diapause (Days 3–4) the ring glands become refractory to PTTH stimulation. Each point represents the mean  $\pm$  SEM of 5–10 separate activation analyses. Results expressed as ecdysone equivalents.

brain equivalent PTTH dose. Figure 5 shows the changing responses of the pupal ring glands as the pupae enter diapause. The initial 3 days show high ecdysone production levels, and therefore a high level of ring gland competency to respond to PTTH (about 2200 pg/4 h). As the pupae entered diapause between day 3 and day 4 after pupariation, the glands became refractory to PTTH stimulation, and the level of ecdysone production fell to the limit of detection. The glands remained refractory until development resumed following the whole pupa being subjected to a period of warming (Richard and Saunders, unpublished observations).

When the competency of the diapause destined ring gland from *Calliphora* larvae was followed (Fig. 6), the initial ecdysone production level in post-feeding larvae was about 900 pg/4 h. This level fell between

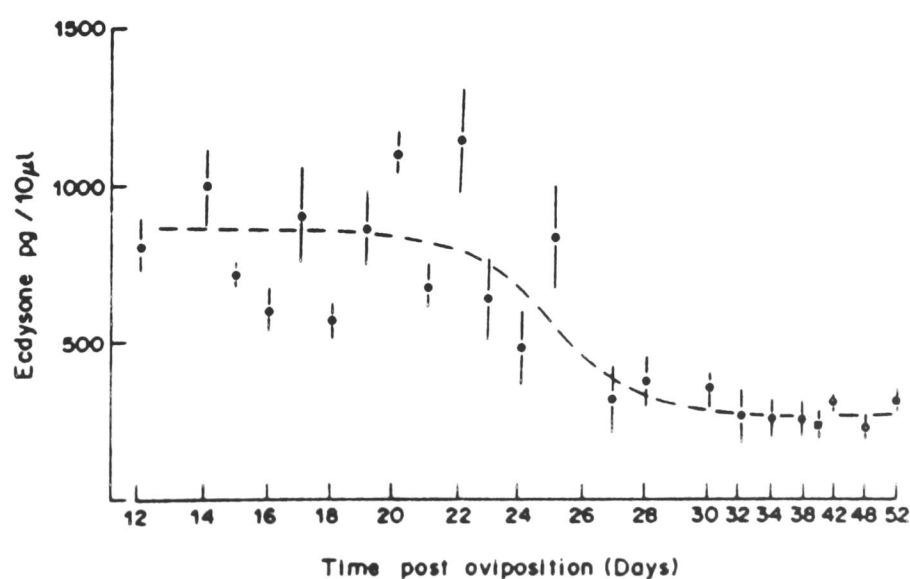


Fig. 6. The competency of ring glands, taken from diapause-destined *Calliphora* larvae (maintained at 11°C in darkness) to synthesise ecdysone in response to incubation with PTTH (0.5 brain equivalents *Sarcophaga* 4 h prepupal brain extract) in Grace's medium plus 10 mM  $\text{CaCl}_2$ , as the larvae entered diapause (●---●). The basal level of ecdysone release by unstimulated ring glands (■). Each point represents the mean  $\pm$  SEM of 5–9 separate activation analyses. Results expressed as ecdysone equivalents.

day 22 and 28 after oviposition, as the larvae entered diapause, indicating either a more gradual loss of competency than in *Sarcophaga* or a lack of synchrony within the population, or both. The ring glands continued to produce ecdysone at a basal level of about 250 pg/4 h with no elevation by PTTH stimulation. These results contrast with those from *Sarcophaga* which showed a very rapid transition into diapause and a complete absence of residual basal competency. The level of circulating ecdysteroids in the haemolymph of diapausing *Calliphora* (Fig. 7) are very low, close to the limit of detection.

#### The recovery of ring gland competency in *Calliphora* larvae

Figure 7 also demonstrates the rapidity with which diapausing *Calliphora* larvae can respond to a temperature increase from 11 to 25°C in constant darkness. Day-52 larvae regained normal ring gland competency within 24 h and all had pupariated within 36 h. There was a degree of spread of pupariation times between 12 and 36 h after the temperature change. Those larvae maintained at 11°C, continued to produce basal levels of ecdysone regardless of PTTH stimulation. Similar recovery data were obtained from day-58 diapausing *Sarcophaga* pupae transferred from 11 to 25°C. All had broken diapause and commenced adult development within 36 h (Richard and Saunders, unpublished observations). Brain-ring gland complexes from diapausing *Calliphora* larvae did not regain competency to respond to PTTH stimulation following a temperature increase from 11 to 25°C *in vitro* (Table 1). Only when the brain-ring gland complex was *in vivo* for the warming period did the ring gland respond by regaining competency. The ring glands removed from diapausing brain-ring gland complexes incubated at 25°C for 24 h continued to secrete ecdysone at the basal level.

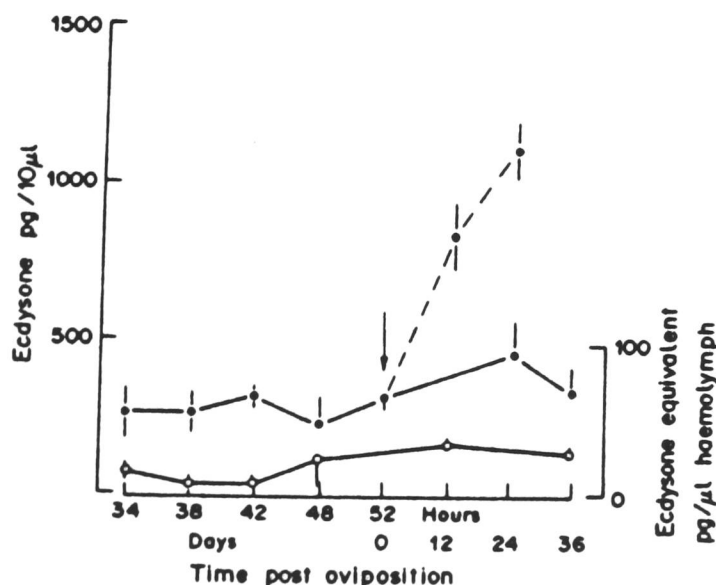


Fig. 7. The competency of diapausing *Calliphora* larval ring glands to respond to PTTH (0.5 brain equivalents *Sarcophaga* 4 h prepupal brain extract) in Grace's medium plus 10 mM CaCl<sub>2</sub> when maintained at 11°C (●—●). At day 52/0 h (arrow) a group of larvae were transferred to 25°C and tested with PTTH (●---●). The level of circulating ecdysteroids in the haemolymph at 11°C is shown (○—○). Each point represents the mean  $\pm$  SEM of 5–7 separate activation analyses. Results expressed as ecdysone equivalents

Table 1. The effects of a period of warming (24 h at 25°C) on the *in vitro* ability of ring glands from diapausing *Calliphora vicina* to respond to 0.5 brain equivalents of *Sarcophaga* prothoracicotrophic hormone

Treatment	PTTH (0.5 b.e.)	Blank (No PTTH)
Brain-RG warmed	339 $\pm$ 120	325 $\pm$ 87
Whole animal warmed	1137 $\pm$ 100	281 $\pm$ 86
Whole animal unwarmed	326 $\pm$ 33	315 $\pm$ 107

Results expressed as pg ecdysone secreted per ring gland per 4 h at 25°C. Each figure is the mean  $\pm$  SEM of 5–9 separate activation analyses. Results expressed as ecdysone equivalents.

b.e. = brain equivalents; RG = ring gland; PTTH = prothoracicotrophic hormone.

#### The levels of PTTH in diapausing *Sarcophaga* brains

It has been established that the prothoracic glands of *Sarcophaga* and *Calliphora* lose their *in vitro* competency to respond to PTTH stimulation when in diapause. The question of whether PTTH synthesis or storage is altered in diapause is addressed in Fig. 8. This dose-response curve for post-feeding *Sarcophaga* larval ring glands to day-35 post-pupariation *Sarcophaga* diapausing brain extracts indicates that similar levels of PTTH are maintained in diapausing brains as in non-diapause-destined prepupal brains (Fig. 1). The level of PTTH in 96 h post-pupariation diapause-destined pupal brains is also comparable with the non-diapause-destined level.

#### DISCUSSION

The present investigation has focussed on a number of elements in the endocrinology of diapause in two dipteran species. Much of the work presented here is based on previous investigations into prothoracic gland function in other species, notably *Sarcophaga bullata* (Roberts *et al.*, 1984) and *Manduca sexta* (Smith *et al.*, 1984, 1985, 1986).

The *in vitro* assay methodology was based on that developed by Bollenbacher *et al.* (1979) and Roberts *et al.* (1984) in which ring glands, containing the

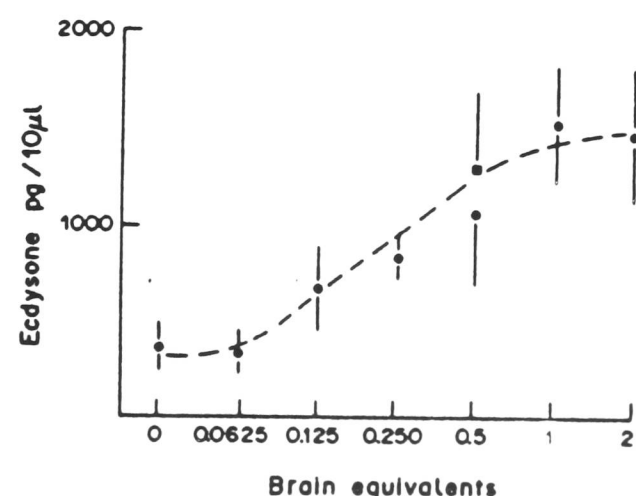


Fig. 8. Dose-response curve for brain extracts from day-35 post-pupariation diapausing *Sarcophaga* pupae (maintained at 18°C) on post-feeding *Sarcophaga* larval ring glands (●). The closed square (■) shows the effect of a 0.5 brain equivalent dose prepared from a 96 h post-pupariation (diapause-destined) pupal brain. Each point represents the mean  $\pm$  SEM of 5–10 separate activation analyses. Results expressed as ecdysone equivalents.

prothoracic glands, were incubated in brain extracts from 4 h non-diapause-destined prepupae. They noted that ring glands from different developmental stages of larvae and pupae secreted ecdysone at different rates *in vitro*. These rates corresponded roughly with rises and falls in haemolymph ecdysteroid titres *in vivo*. Ring glands from post-feeding larvae were chosen in this study as these gave the most reliable indication of prothoracic gland response to stimulation, presumably because they had not recently been stimulated *in vivo*. When ring glands from red-spiracled larvae, the stage immediately prior to pupariation, were used, more ecdysone was produced since these glands had already been stimulated by endogenous PTTH. The dose-response curves obtained in this investigation are similar to those reported by Roberts *et al.* (1984) working with *S. bullata* except that the maximal activation they reported was four times higher than that reported here. This may be due to a species difference or to an undetermined difference in experimental protocol. The degree of activation here was more comparable with that reported by Bollenbacher *et al.* (1979) for *Manduca sexta* when an activation ratio (amount produced by activated glands/amount released by unactivated glands) of 4 was reported. Roberts *et al.* (1984) reported an activation ratio of approx 11.

To investigate the involvement of a cyclic nucleotide secondary messenger system in the action of PTTH, isolated ring glands were incubated with agents reported to raise the intracellular levels of cAMP. The effects of these concentrations of cAMP analogues were similar to those reported by Smith *et al.* (1984) in *Manduca sexta*. They reported that an increase in the intracellular levels of cAMP can mimic the steroidogenic action of PTTH. That dibutyryl cAMP stimulated a greater response than 8-bromo cAMP in *Sarcophaga* agrees with the situation reported in *Manduca* pupal prothoracic glands *in vitro* (Smith *et al.*, 1984). However, these workers reported that there was little difference in activation by the two nucleotides in larval prothoracic glands. By inhibiting the catalysis of cAMP in the prothoracic gland, MIX can bring about an accumulation of intracellular cAMP, associated with the increased synthesis and production of ecdysone noted in the present study. That the PTTH-saturated level could be increased by the action of MIX implies that despite receptors being saturated, the glands themselves were not maximally releasing ecdysone.

No steroidogenic effect was noted with the cyclic guanosine monophosphate analogues dibutyryl cGMP and 8-bromo cGMP; the ring glands continued to secrete ecdysone at the basal level. This agrees with the findings of Smith *et al.* (1984) who noted no activation by cGMP analogues in *Manduca*. Denlinger and Wingard (1978) reported that cGMP and its derivatives injected into diapausing pupae effected recovery from diapause by *S. crassipalpis* whereas cAMP had no such effect. That cGMP has no stimulatory effect on isolated *M. sexta* prothoracic glands or *S. argyrostoma* ring glands suggests that diapause recovery may be initiated outside the ring gland complex.

The involvement of extracellular calcium in PTTH

action was indicated in *Sarcophaga* by the addition of the chelating agent EDTA to the PTTH activation medium. Upon subsequent incubation of the ring glands, there was no increase from the basal level of ecdysone secretion. Smith *et al.* (1985) reported a similar effect in *Manduca* where extracellular calcium was required for PTTH action. They reported that basal ecdysone production or cAMP or MIX activation would occur in the presence or absence of calcium. That the calcium ionophore A-23187, which acts by increasing the permeability of biological membranes to divalent cations (Pfeiffer *et al.*, 1978), was able to increase the intracellular level of cAMP in *Manduca* prothoracic glands suggests that calcium and cAMP have a sequential role in PTTH-activated steroidogenesis (Smith *et al.*, 1985). Smith and Gilbert (1986) have proposed that PTTH acts by increasing the influx of extracellular calcium, which in turn enhances the formation of cAMP by a calcium ion-sensitive adenylate cyclase system. The cAMP may then act by activating a cAMP-dependent protein kinase responsible for the phosphorylation of a rate-limiting enzyme in the synthesis of ecdysone.

Ring glands taken from post-feeding *Calliphora* larvae were subjected to similar treatments to those from *Sarcophaga*. It is of particular interest that *Calliphora* ring glands respond to a crude extract of PTTH from *Sarcophaga* brains. Similar cross reactivities have been demonstrated between *M. sexta* and *S. bullata* (Roberts *et al.*, 1985) and between *M. sexta* and mosquitoes (Gilbert, pers. comm.). However, although crude brain extracts are effective in the latter case, purified preparations are not. Nevertheless, it seems possible that the mechanisms of PTTH action are very similar across a wide range of insect species and orders.

It would appear that diapausing ring glands from *Sarcophaga* have lost the ability to respond to PTTH via the calcium influx mechanism or to cAMP. These results strongly support Smith *et al.* (1986) who suggested that the cellular basis of PTTH refractoriness in diapause occurs beyond the level of cAMP action, possibly at the level of cAMP-dependent protein kinase activity, even though diapausing *Manduca* prothoracic glands synthesized more cAMP in response to PTTH or A-23187 stimulation than non-diapausing glands. Richard *et al.* (1986) demonstrated that the levels of circulating ecdysteroids in diapausing *Sarcophaga* pupae were very low, presumably because of the refractory nature of the ring glands.

Ring glands from *Calliphora* do not lose their basal level of ecdysone production as they enter diapause. However, the glands become refractory to PTTH and dibutyryl cAMP stimulation. This implies that steroidogenic control may become uncoupled from PTTH/cAMP mediation in diapause. A prothoracicotropic factor distinct from PTTH, such as that proposed by Watson *et al.* (1985), may be involved which sustains ecdysone synthesis without associated cAMP formation (Smith *et al.*, 1986). Despite the higher basal rate of synthesis of ecdysone in diapausing *Calliphora* ring glands *in vitro*, the haemolymph ecdysteroid titre is comparable with that of *Sarcophaga* (Richard *et al.*, 1986). The ecdysone



metabolism and excretion pathways may therefore still be in operation during diapause in *Calliphora*.

Larvae of *C. vicina* have previously been described as being in diapause if they have not pupariated within 32 days of the hatching of the larvae at 12.5°C (Vinogradova and Zinovjeva, 1972). The results for ring gland competency presented here suggest that these larvae enter diapause between day 22 and day 28 after oviposition. This is unlike the rapid loss of *Sarcophaga* ring gland competency. *Calliphora* larvae do not enter diapause at an easily identifiable stage like *Sarcophaga* pupae which do so after the formation of the phanerocephalic pupa (Fraenkel and Bhaskaran, 1973). This coupled with the physiological asynchrony within the *Calliphora* population after 22 days may account for the wider time-course of ring gland competency changes in *Calliphora*. It would appear that the 32-day criterion as suggested by Vinogradova and Zinovjeva (1972) for larval diapause would account for the majority of individuals at these temperatures.

This investigation showed that temperature increases administered to isolated *Calliphora* brain–ring gland complexes *in vitro* would not restore ring gland competency to PTTH stimulation; only the warming of the whole animal would do this. Smith *et al.* (1986) proposed that recovery from diapause may be dependent upon the release of trophic factors (e.g. PTTH) that ultimately stimulate steroidogenesis. However, if PTTH were the required trophic factor for recovery, one may have expected the *Calliphora* brain–ring gland complexes to recover after warming *in vitro*. Smith *et al.* (1986), however, also proposed that ecdysone synthesis may be reinitiated through the action of a haemolymph prothoracicotrophic factor (Watson *et al.*, 1985), which is apparently similar to a factor produced by the *Manduca* fat body in response to juvenile hormone stimulation (Gruetzmacher *et al.*, 1984a, b). A similar non-cerebral factor may therefore be involved in this recovery in *Calliphora*. Alternatively, the temperature-sensing apparatus may be located outside the brain–ring gland complex therefore requiring the rest of the body for complete ring gland recovery. The interruption of diapause in *S. crassipalis* by the injection of cGMP and its derivatives (Denlinger and Wingard, 1978) may be involved in this process of recovery although cGMP has little steroidogenic effect on isolated *Sarcophaga* and *Manduca* prothoracic glands. Evidence now points to the suggestion that although diapause induction may be a brain centred event, diapause termination is not. The involvement of the rest of the body appears to be necessary and is possibly associated with the action of cGMP and a non-cerebral factor, perhaps that suggested by Watson *et al.* (1985).

Bowen *et al.* (1984a) suggested that diapause in *Manduca* resulting from the failure of the prothoracic glands to secrete ecdysone may be in response to both the development of refractoriness to PTTH stimulation and to the curtailment of PTTH release, as opposed to its synthesis and transport. This view is supported by the data presented here from *Sarcophaga* which show that prediapause and day-35 post-pupariation diapausing pupal brains contain comparable amounts of PTTH to non-diapause destined

prepupal brains. These levels would presumably be adequate for normal adult development to continue were the PTTH released, and the ring gland competent. It appears that PTTH is present in the brains of most pupal diapausing species during diapause. In one species, *Hyalophora cecropia* (Williams, 1968) PTTH has been shown to absent during diapause; it must be synthesized prior to recovery from diapause.

In conclusion, it would appear that ring gland function in *Sarcophaga argyrostoma* and *Calliphora vicina* is under the control of a similar PTTH-activation mechanism. This may work via a calcium-dependent cAMP-mediated secondary messenger system. Ring gland competency to produce ecdysone is impaired in diapause in both species apparently at a level beyond cAMP action. Diapausing *Sarcophaga* ring glands do not appear to secrete ecdysone, but those from diapausing *Calliphora* continue to secrete basal levels independently of the PTTH/cAMP system. The time-course of these competency changes differs between the two species. In *Sarcophaga*, diapause, or the change in competency, appears to be more closely associated with a particular developmental stage than in *Calliphora*. Ring glands from diapausing *Calliphora* and *Sarcophaga* will recover their competency to respond to PTTH stimulation very rapidly following a temperature increase to 25°C. Denlinger (1981) suggested that diapause termination in *Sarcophaga* is a two-part process, consisting of a temperature-insensitive stage followed by a temperature-sensitive stage in which the pupae quickly respond to a rise in temperature. The levels of PTTH in prediapause and diapausing brains of *Sarcophaga* are similar to those in non-diapause-destined pupal brains. Diapause in these two species therefore results from a combination of developing refractoriness to PTTH activation by the ring glands, and a cessation of PTTH release by the brain, but not of its synthesis and storage. Diapause termination however, can be brought about by environmental changes which may restart the release of PTTH and reverse the refractory nature of the ring gland. PTTH appears not be involved in the actual initiation of development from the diapause state; a non-cerebral factor, possibly a peptide associated with the action of cGMP, may be important. The data suggest that despite similarities in the mechanisms of diapause in the two species, there may be differences in the depth of diapause experienced by *Sarcophaga* and *Calliphora*. This is suggested by the basal levels of ecdysone synthesis in diapausing *Calliphora* larvae and by the ease of recovery by these larvae when compared with diapausing *Sarcophaga*.

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IN VITRO ACTIVATION OF PROTHORACIC GLANDS FROM  
DIAPAUSE AND NON-DIAPAUSE DESTINED *SARCOPHAGA*  
*ARGYROSTOMA* AND *CALLIPHORA VICINA*

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The in vitro activation of isolated prothoracic glands from *Sarcophaga argyrostoma* (Robineau-Desvoidy) to produce ecdysone was accomplished in a dose related manner (Figure 1) with extracts of prepupal brains, presumably containing prothoracicotropic hormone (PTTH) (Roberts et al., 1984). Ecdysone was measured according to Richard and Saunders (1986). The steroidogenic action of PTTH was mimicked by dibutyryl cAMP, and the phosphodiesterase inhibitor MIX (Figure 2), suggesting the involvement of a cyclic nucleotide mediated secondary messenger system in PTTH action (Smith et al. 1984).

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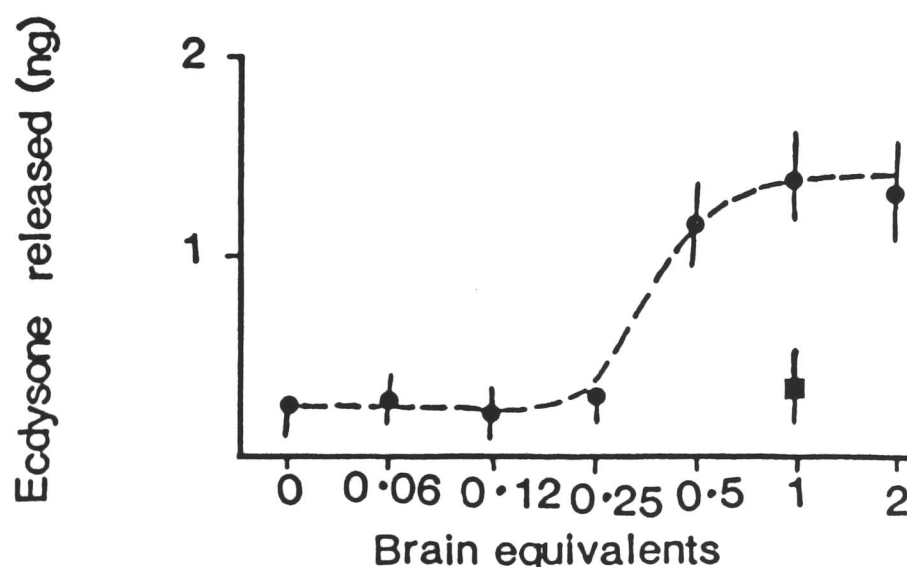


Figure 1: Dose response curve for the in vitro activation of *Sarcophaga* post-feeding larval (●---●) ring glands incubated with PTTH extracts from 4h prepupal brains in Grace's medium plus 10mM CaCl<sub>2</sub> for 4h at 25°C. An extract of ventral ganglion was used as a control (■). Each point represents the mean  $\pm$  SEM of 5 to 10 separate

activation analyses. Results expressed as ecdysone equivalents.

The removal of calcium ions by EDTA reduced PTTH action, as noted by Smith *et al* (1985) in *Manduca sexta*. Cyclic GMP and its derivatives had no effect on ecdysone release. PTTH extracted from *Sarcophaga* and dibutyryl cAMP were found to be effective at stimulating ecdysone release by isolated ring glands from *Calliphora vicina* (Meigen) larvae. Roberts *et al* (1986) showed that PTTH from *Sarcophaga* and *Manduca* could stimulate ecdysone release interspecifically, and suggested a great degree of uniformity in PTTH structure and function in insects.

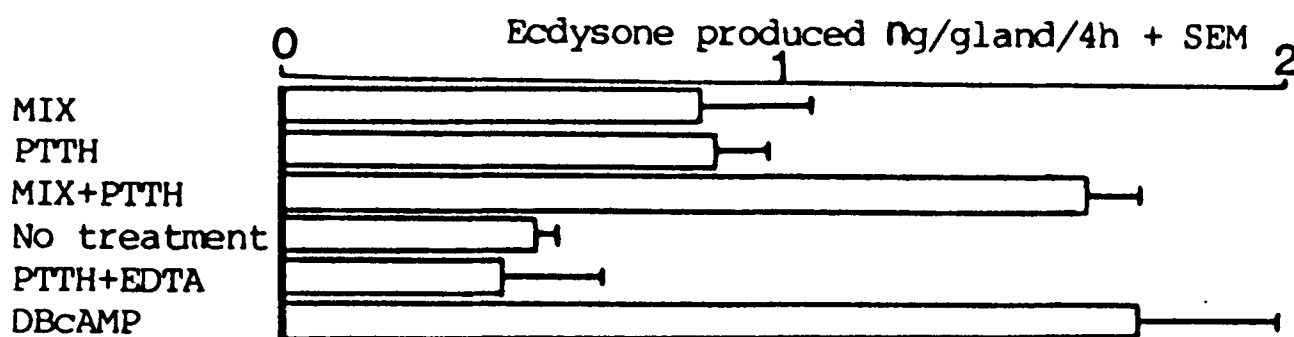


Figure 2: The effects of 0.1mM 1-methyl-3-isobutylxanthine (MIX) and 10mM EDTA on the action of PTTH (0.5 brain equivalents) and the effect of 10mM dibutyryl cAMP on *Sarcophaga* post-feeding larval ring glands *in vitro*. Each figure is the mean  $\pm$  SEM of 6 to 10 separate activation analyses. Results expressed as ecdysone equivalents.

*Sarcophaga argyrostoma* enters pupal diapause in winter (Fraenkel and Hsiao, 1968), after the formation of the phanerocephalic pupa. Diapause in this species is induced by short day photoperiods experienced by the developing embryo and larva (Denlinger, 1971). *Calliphora vicina* however, enters a larval diapause induced principally by short days experienced maternally (Vinogradova and Zinovjeva, 1972). In the diapause state, both species display a very low haemolymph ecdysteroid titre (Richard *et al*, 1986; Richard and Saunders, 1986), presumably as a result of neuroendocrine regulation, which interrupts the release of PTTH from the brain/retrocerebral complex (Bowen *et al*, 1984). As the *Sarcophaga* population entered pupal diapause at 96h post-pupariation at 18°C, the prothoracic glands became refractory to PTTH, taking 24h to lose their competency to respond (Figure 3A). The levels of

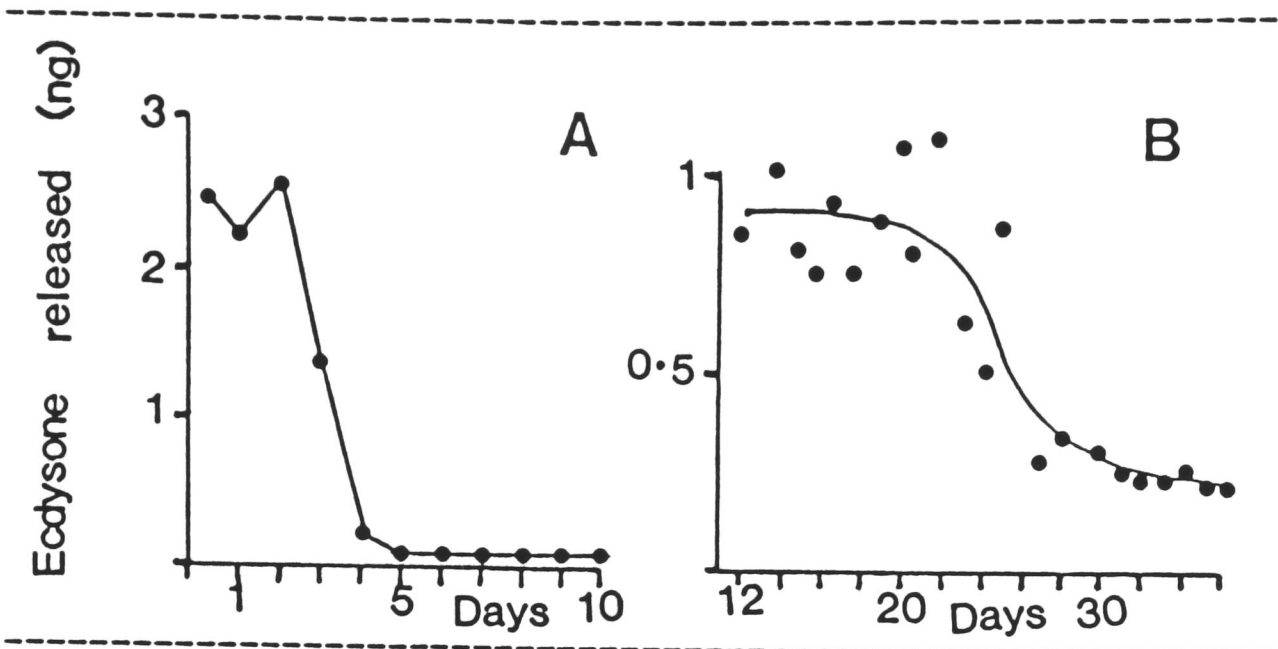


Figure 3: The competency of ring glands taken from post-pupariation diapause destined Sarcophaga (maintained under LD12:12 at 18°C) (A), and ring glands taken from post-wandering diapause destined Calliphora larvae (maintained at 11°C) (B) to synthesise ecdysone in response to incubation with 0.5 brain equivalents of 4h Sarcophaga prepupal brain extract.

ecdysone released in diapause were below the limits of detection of the radioimmunoassay used. The glands could not be stimulated by the cyclic nucleotides indicating that the block to PTTH action occurred beyond the proposed stage of cyclic nucleotide mediation. The Calliphora population, which has a larval diapause, took six days to reach this state (Figure 3B). Unlike Sarcophaga, their prothoracic glands continued to release ecdysone at a basal rate refractory to stimulation by PTTH or cyclic nucleotides. Following a temperature increase from 11°C to 25°C, all diapausing Calliphora had regained prothoracic gland competency within 24h and had pupariated within 36h (Figure 4). When isolated brain/ring gland complexes from diapausing Calliphora were incubated at 25°C, no recovery of ring gland competency was noted, indicating the requirement for in vivo reactivation after diapause. This suggested a different mechanism for diapause termination than for diapause induction. The levels of PTTH in pre-diapause and day 35 post-pupariation diapause brains of Sarcophaga were shown to be similar to those of non-diapause destined prepupal brains.

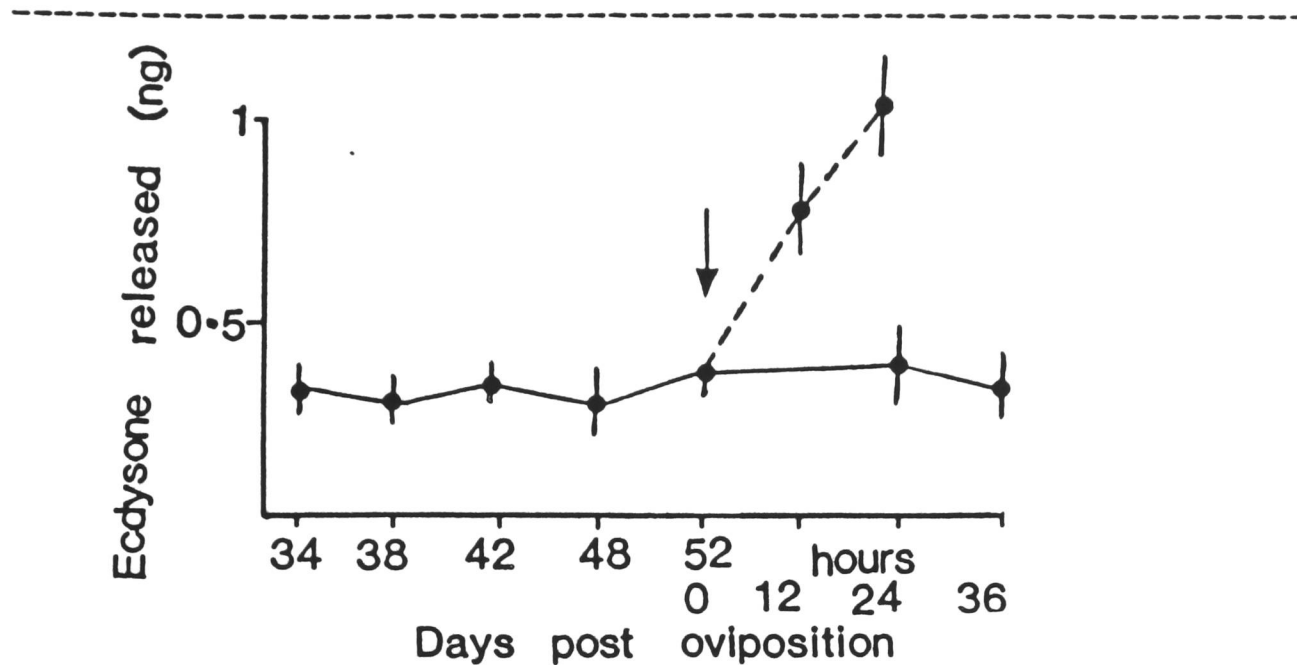


Figure 4: The competency of diapausing Calliphora larval ring glands to respond to PTH when maintained at 11°C (●—●). At day 52/0h (arrow) a group of larvae were transferred to 25°C and tested with PTH (●---●).

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## HAEMOLYMPH ECDYSTEROID TITRES IN DIAPAUSE- AND NON-DIAPAUSE- DESTINED LARVAE AND PUPAE OF *SARCOPHAGA ARGYROSTOMA*

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**Abstract**—No differences were observed between the rates of development of larvae and pupae from diapause- and non-diapause-destined lines of *Sarcophaga argyrostoma* except that those destined for diapause have a longer post-feeding, wandering, larval phase associated with a lower haemolymph ecdysteroid titre, as measured by radioimmunoassay. Following pupariation, both cultures show a high haemolymph titre associated with larval/pupal apolysis. The developing culture displays an ecdysteroid peak at 72 h after pupariation which may be involved with pupal/adult apolysis and the initiation of pharate-adult development. This peak is reduced in the diapause-destined culture. Following the initiation of pharate adult development, there is a very large peak at 85–90 h. Those pupae entering diapause display very low titres as a result of the failure of the brain/prothoracic gland axis to release ecdysone. There are no quantitative or qualitative differences between the titres of specific ecdysteroids in the prepupae of the two lines as determined by reverse-phase high-performance liquid chromatography. A preliminary examination of the levels of free and conjugated ecdysteroids has provided the basis for proposing a mechanism of ecdysone metabolism in this insect.

**Key Word Index:** *Sarcophaga argyrostoma*, diapause, ecdysteroids

### INTRODUCTION

Large seasonal variations in temperature, humidity and food availability occur in many regions of the world, particularly at higher latitudes. To cope with periods when conditions are unsuitable for continuous development, many insects enter a diapause state in which growth is arrested and metabolism reduced. The onset of diapause is frequently determined by day length, an environmental feature which is a precise and reliable indicator of the seasons. The flesh fly *Sarcophaga argyrostoma* (Robineau-Desvoidy), which enters pupal diapause in winter (Fraenkel and Hsiao, 1968a), exhibits a temperature-modified photoperiodic response such that long days promote continuous development and short days coupled with lower temperatures induce diapause (Saunders, 1981). Dörlinger (1971) showed that the embryonic and early larval stages of *Sarcophaga* are the most sensitive to photoperiod, although diapause does not occur until the pupal stage.

The endocrinology of pupal diapause is not fully understood, although Williams (1952), working with *Hyalophora cecropia*, suggested that it may result from the inactivation of the brain/prothoracic gland system. This view is supported by Bowen *et al.* (1984a) who showed that in the diapausing tobacco hornworm *Manduca sexta*, the prothoracic glands become refractory to prothoracicotropic hormone

(PTTH) stimulation. They further suggested that PTTH release by the brain was curtailed. Bowen *et al.* (1985) have shown that there are no differences in the titres of ecdysteroids and juvenile hormones between pre-diapause and non-diapause development in *Manduca*. They concluded that the induction of photoperiodic diapause is probably a brain-centred phenomenon concerned with the release of PTTH. Further evidence for the central role of the brain comes from Bowen *et al.* (1984b) who showed that diapause could be averted in *Manduca* by the implantation of a brain which had been kept under long-day conditions *in vitro*. Diapause in *Manduca* may therefore involve two processes, the restriction of the release of PTTH by the brain, and the prothoracic gland becoming refractory to PTTH stimulation. In the consequent absence of ecdysteroids, growth and development cease.

Wentworth *et al.* (1981) have followed the release of ecdysteroids throughout the larval, intrapupal and adult stages of *S. bullata*. They were able to relate ecdysteroid peaks, as measured by radioimmunoassay (RIA), to physiological and behavioural events such as larval moulting, larval exodus from the food, puparium formation and pharate-adult development.

This paper will concentrate on the haemolymph ecdysteroid titres of larval and pupal cultures of *Sarcophaga argyrostoma* which were raised under diapause- (light:dark 12:12) and non-diapause- (light:dark 18:6) inducing photoperiods. Data for total ecdysteroid titres and specific ecdysteroid titres will be presented.

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## MATERIALS AND METHODS

### Culture maintenance

Stock cultures of *S. argyrostoma* were raised at 25°C under continuous light. The adults were supplied with sugar and water *ad libitum*, and with a daily supply of fresh beef muscle. Newly deposited larvae were transferred on pieces of meat to a supplementary diet made from yeast, dried milk, agar and water cast in plastic dishes. Fully fed larvae were allowed to wander into dry sawdust to form puparia (Saunders, 1971).

### Larval and pupal ecdysteroid titres in diapause- and non-diapause-destined *Sarcophaga*

Experimental cultures were established by allowing adult flies to emerge into either light:dark 12:12 or 18:6 at 25°C. Since intrauterine (and subsequent larval) development occurred in these conditions, the former gave a high incidence of pupal diapause (Saunders, 1982) whilst the latter resulted in its total absence. Twenty-four hours prior to estimated larviposition, the meat was removed from the cages followed by the reintroduction of the meat, 24 h later, for 2 h. This provided a large number of newly deposited larvae with a narrow age range. Three cultures of approx. 300 larvae were established in each photoperiod from the appropriate adult cage. The middle of the 2 h larviposition period was designated time zero, and all larval time points were measured from this. At time zero, and at 4 h intervals thereafter, five larvae were removed from each culture until the onset of pupariation. The larvae were weighed and examined to determine larval instar and the times of larval moults. In this study, changes in the morphology of the posterior spiracles were used as criteria for the determination of the time of larval moults (Saunders, 1986). In the first 24 h of the investigation, the larvae were too small for haemolymph to be collected, so a pooled, whole body, cold methanol extract was made. Subsequently, a volume of haemolymph, between 10 and 20 µl, was taken from each larva and added to nine volumes of cold methanol, the samples being stored at -20°C until analysis.

Following larval exodus, the larvae were placed individually in small tubes containing dry sawdust to form puparia. These newly formed puparia were collected at 4 h intervals, and subsequent puparial samples timed from this point. As pupal and pharate-adult development commenced, the haemolymph became contaminated with large quantities of dissociated fat body cells which were removed by centrifugation prior to analysis by radioimmunoassay.

The diapause incidences of the long-day and short-day lines were checked 10 days after the formation of the puparia.

### Haemolymph ecdysteroid analysis

Total haemolymph ecdysteroid titres were determined on aliquots of larval haemolymph (25 µl) or pupal (20 µl) extracts by a radioimmunoassay (RIA) employing an antiserum (D20-30) that bound ecdysone and 20-hydroxyecdysone equally, but was not useful for measuring side-chain oxidised or conju-

gated ecdysteroid metabolites (Gilbert *et al.*, 1977). [<sup>3</sup>H]ecdysone (60 Ci/mmol; New England Nuclear) was used as the tracer and titres were expressed in ecdysone equivalents. The antibody-bound fraction was precipitated by a second antibody (donkey anti-rabbit immunoglobulin-G; Scottish Antibody Production Unit) and counted in Ultrafluor scintillation fluid (National Diagnostics) in a Packard 2425 Tricarb liquid scintillation counter.

### Specific ecdysteroid analysis

(a) *Sample preparation.* Haemolymph samples from 4, 8 and 12 h puparia, either diapause- or non-diapause-destined, were pooled and extracted with three volumes of methanol. Following centrifugation, the residues were further extracted with 50% aqueous methanol, and partitioned against hexane to remove non-polar lipids. The diapause and non-diapause samples were evaporated and taken up in high-performance liquid chromatography buffer (2.4 ml, 20 mM Tris/perchlorate, pH 7.2) and applied to primed C18 Sep Paks (Waters). After a 2 ml buffer wash, the ecdysteroids were eluted with 7 ml of methanol. The methanol was then evaporated and the residues dissolved in buffer (1 ml) and the samples centrifuged immediately prior to HPLC analysis.

(b) *HPLC conditions.* A Waters HPLC system (two 6000 A pumps, 720/730 system integrator and controller, Schoeffel UV detector (242 nm) and an LKB fraction collector) was employed for ion suppression, reverse phase chromatography (IS-RPHPLC) using a 5 µm Resolve (Waters) C18 column (15 cm × 4.6 mm int. diam.). The solvent system for gradient elution was: Solvent A (5% acetonitrile, 95% 20 mM Tris/perchlorate buffer, pH 7.5), Solvent B (5% buffer, 95% acetonitrile); conditions as indicated in Results section (See Warren and Gilbert, 1986, for details). Fractions (0.5 ml) were collected from which duplicate aliquots (20 µl) were assayed for ecdysteroid immunoreactivity. Two complimentary antisera were employed in the analyses. One (H-22), raised against an ecdysone-22-succinyl-thyroglobulin immunogen, detects ecdysone and its metabolites resulting from phase-1 (oxidation) and phase-2 (conjugation) reactions of the side chain; and H-2 (derived from a 20-hydroxyecdysone-2-succinyl-thyroglobulin immunogen) capable of detecting phase-1 and phase-2 metabolites of the A-ring of ecdysone, e.g. 3- $\alpha$ -epimers and ester conjugates at C-2 and C-3 (Warren and Gilbert, 1986). Assay composition and termination by protein A were according to Warren and Gilbert (1984).

(c) *Ecdysteroid hydrolysis.* Following IS-RPHPLC, fractions containing highly polar products and conjugates (HPP, 0–35 min) were pooled, evaporated (reduced pressure at 35°C) and the residue taken up in 0.05 M acetate buffer, pH 5.0 (10 ml), containing 10 mg each of lyophilised crude esterases from *Helix pomatia* (Sigma H-1) and acid phosphatase from potatoes (Sigma). Following incubation overnight at 37°C, the centrifuged solution (together with a buffer wash of the precipitate) was applied to a primed C18 Sep Pak which was washed (2 ml HPLC buffer) and eluted (7 ml methanol) as before, prior to analysis by IS-RPHPLC.



## RESULTS

The rates of growth were similar in both the long- and short-day cultures (Fig. 1). Both cultures showed a 1000-fold increase in weight over the 72 h period following larviposition. Subsequent post-feeding weight loss by the long-day culture was more rapid than that of the short-day culture, but following larval exodus, those larvae raised under light:dark 12:12 conditions wandered for 24 h longer than those under light:dark 18:6. The two cultures reached the same mean larval weight by the onset of pupariation. The diapause incidences of the long- and short-day cultures were 0 and 89% respectively.

Figures 2A and 2B show the haemolymph ecdysteroid titres obtained under the two photoperiods. Both cultures displayed a high, whole-body ecdysteroid titre at larviposition, and haemolymph peaks prior to each moult. Another large ecdysteroid surge occurred before larval exodus from the food. The two cultures exhibited similar titres except that those larvae raised under light:dark 18:6 had a higher mean post-feeding titre ( $97 \pm 21$  pg/ $\mu$ l) than those raised under light:dark 12:12 ( $48 \pm 8$  pg/ $\mu$ l). A large peak was seen prior to puparium formation in the long-day culture (Fig. 2B), but was not seen in the short-day culture (Fig. 2A), possibly as a result of increased asynchrony of development under this photoperiod.

Figures 3A and 3B show the prepupal, pupal, and pharate-adult ecdysteroid titres timed from the formation of the puparium. Both cultures showed a high titre (about 500 pg/ $\mu$ l) following pupariation, presumably associated with larval/pupal apolysis, and a gradual drop through 24 and 48 h. The titre in the diapause destined culture remained low except for a

small peak (about 100 pg/ $\mu$ l) at 72 h, whereas that in the developing culture rose to twice that level (about 200 pg/ $\mu$ l). This peak may be involved with pupal/adult apolysis and the initiation of pharate-adult development in the non-diapause destined culture. The developing insect titre is further characterized by a large peak (550 pg/ $\mu$ l) at 85–90 h. This peak is presumably associated with unspecified developmental events in the pharate adult.

Few qualitative differences were observed among the haemolymph ecdysteroids in diapause and non-diapause destined *Sarcophaga* prepupae. Figure 4 shows the haemolymph ecdysteroid profile of prepupae raised under light:dark 18:6. The two most prominent peaks correspond to 20-hydroxyecdysone and ecdysone, respectively. The area of RIA-positive activity between fractions 20 and 70 corresponds to

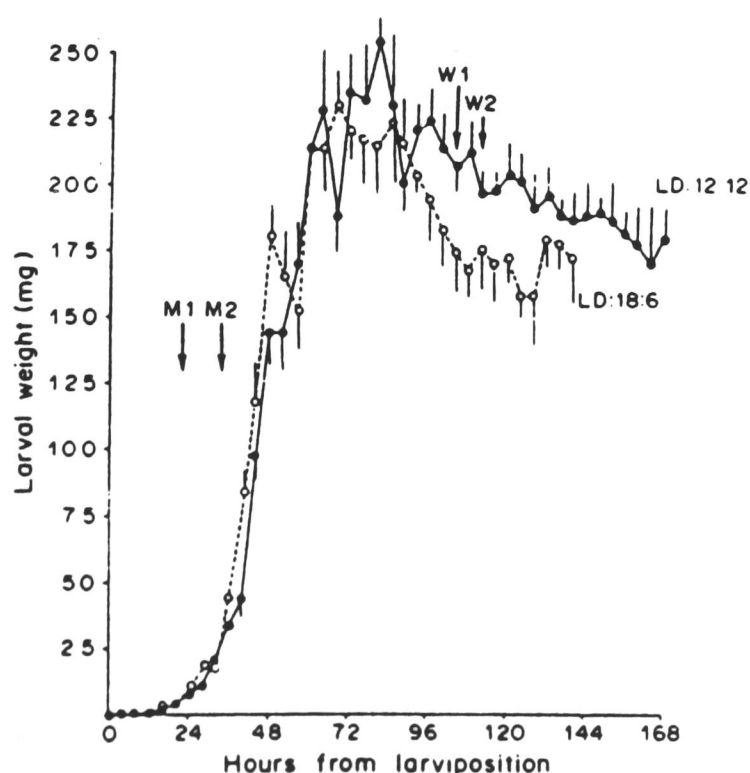


Fig. 1. The growth and development of larval *Sarcophaga argyrostoma* maintained in either light:dark (LD) 12:12 (●—●) or 18:6 (○—○) conditions at 25°C. Each point represents the mean of five weighings  $\pm$  2 SE. M1 and M2 denote the first and second larval moults, respectively. W1 and W2 denote the onset of larval wandering from the food for LD 18:6 and LD 12:12, respectively.

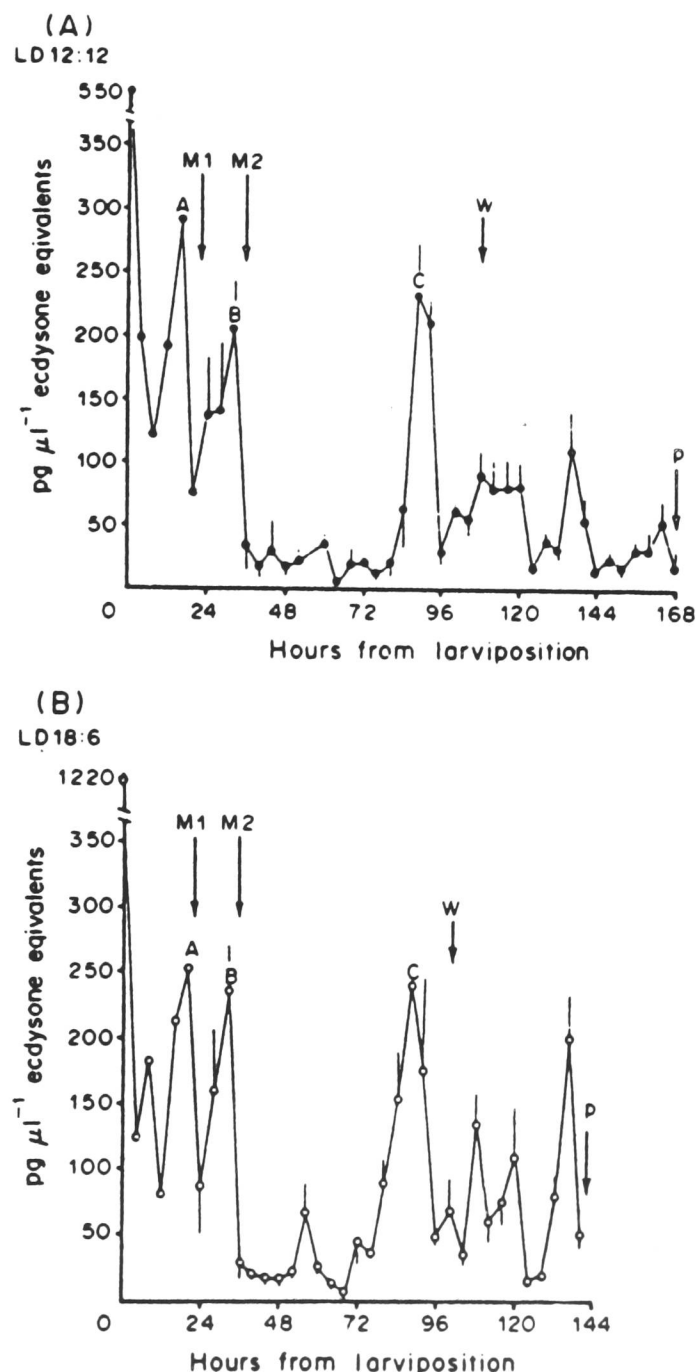


Fig. 2. The ecdysteroid titres of larval haemolymph from cultures raised under (A) light:dark (LD) 12:12 or (B) light:dark (LD) 18:6. A, B and C are peaks associated with larval moults (M1 and M2) and the onset of larval wandering (W). P is the onset of pupariation. Data in the first 24 h of larval development are based on pooled whole body homogenates. Subsequent points represent the mean of five determinations  $\pm$  SEM.

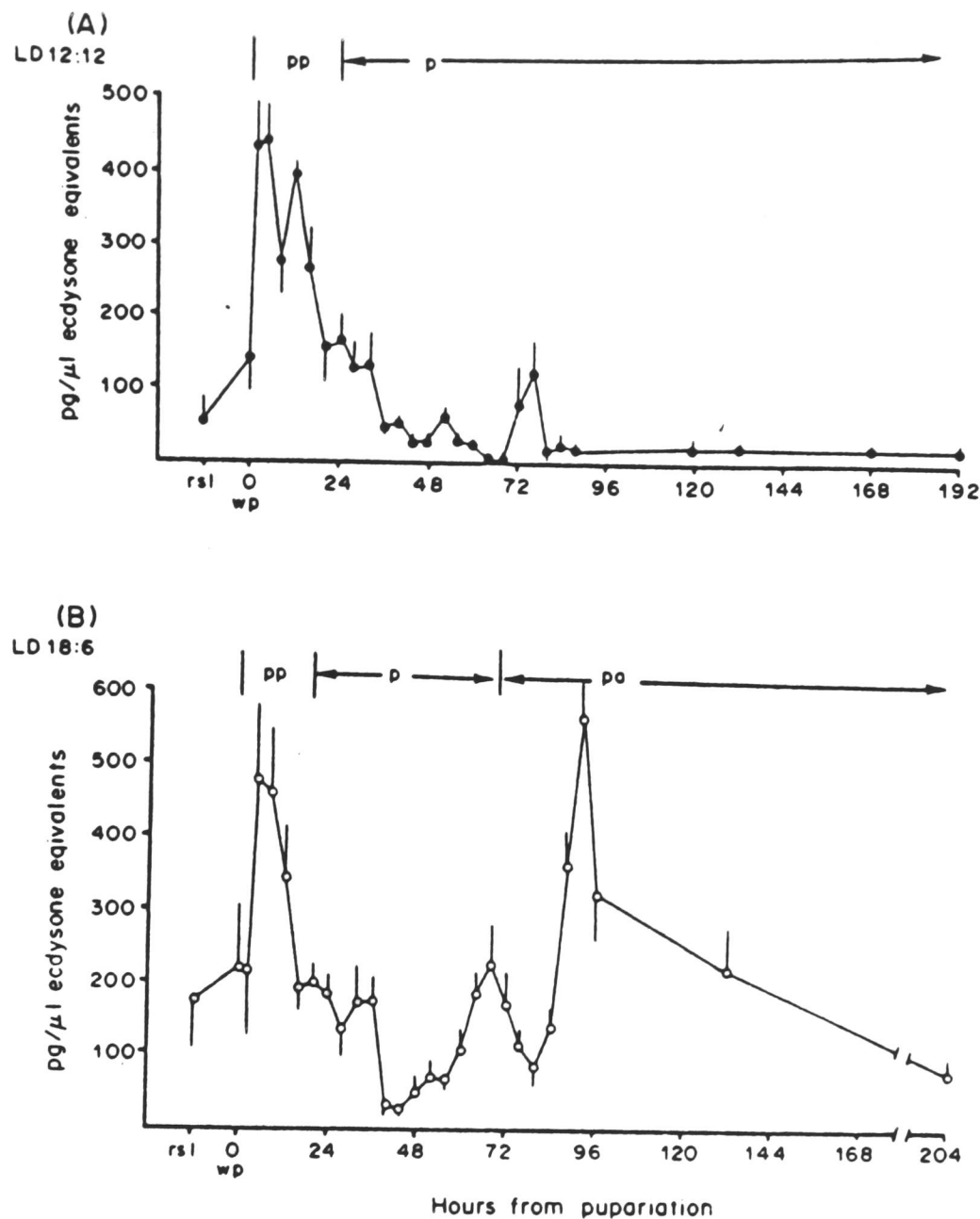


Fig. 3. Pupal haemolymph ecdyst eroid titres under (A) diapause-inductive and (B) non-diapause-inductive photoperiods at 25°C. Each point represents the mean of five determinations  $\pm$  SE. RSL, red spiracled larvae; WP, White puparia; PP, prepupal; P, Pupal; PA, pharate adult.

a series of poorly resolved highly polar products, that may represent side-chain ester conjugates and ecdysoneic acids. These fractions account for approx. 60% of the RIA activity in both diapause- and non-diapause-destined lines. The upper trace (H-2 antiserum) supports the identification of these ecdysteroids by its sensitivity to 20-hydroxyecdysone and ecdysone, but not to the other components, ruling out 3- $\alpha$ -epimers or A-ring conjugates. The concentrations of the free ecdysteroids from diapause- and non-diapause-destined lines are shown in Table 1. No major differences between the levels of each ecdysteroid identified were observed. These ecdysteroids have not been identified unequivocally because the data are based on analyses by reverse-phase HPLC only. Absolute identification would require analysis by normal-phase HPLC or preferably by mass spectroscopy.

Figure 5 shows the ecdysteroid profile of the hydrolysed HPP fractions from the pooled light:dark 18:6 and light:dark 12:12 samples. The levels of conjugated ecdysone and 20-hydroxyecdysone are much lower than in the free ecdysteroid fractions

(Table 2). The conjugated levels of 20,26-dihydroxyecdysone are approx. five times higher than the free levels, and the levels of 26-hydroxyecdysone are forty times higher. A significant level of makisterone A was noted in the highly polar products conjugate fraction. The position of the highly polar 20-hydroxyecdysoneic acid did not change after the deconjugation procedures which suggested that it was not subject to any conjugation.

It would appear that the overall levels of ecdysteroids in the diapause- and non-diapause-destined lines of *Sarcophaga* are similar, and that no significant differences were noted in the levels of specific detectable ecdysteroids.

#### DISCUSSION

The development and moulting of *Sarcophaga argyrostoma* is controlled by the endocrine system. A moult occurs in response to a pulse of 20-hydroxyecdysone in the haemolymph, although the precise mechanism of its action is not fully understood. The two larval-larval moults typical of

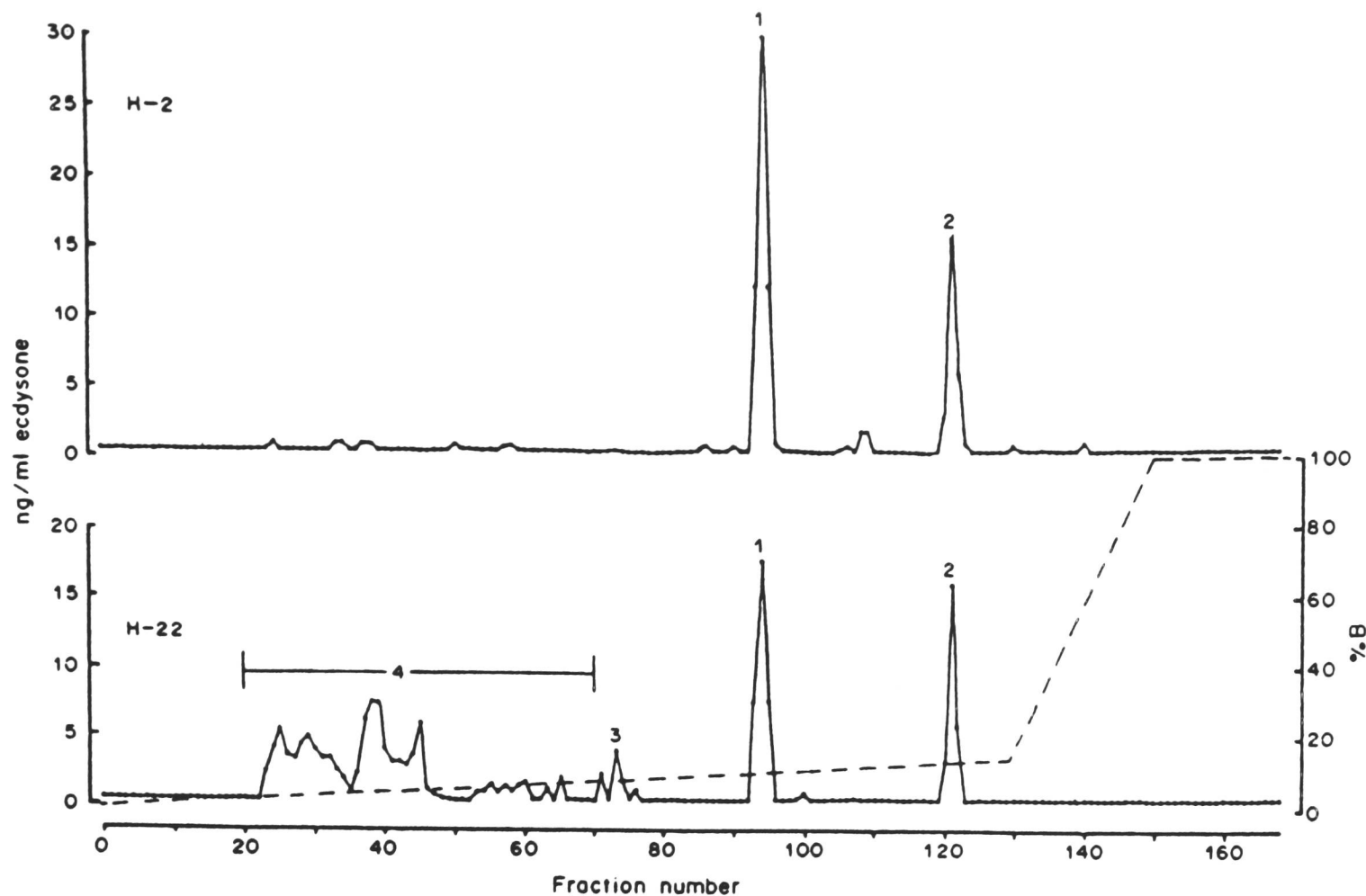


Fig. 4. IS-RPHPLC trace of free ecdysteroids extracted from pooled haemolymph samples from 4, 8 and 12 h non-diapause-destined puparia. (1) 20-hydroxyecdysone; (2) ecdysone; (3) 20,26-dihydroxyecdysone; (4) highly polar ecdysteroid products. Ecdysteroids quantified by radioimmunoassay (RIA) on collected fractions with H-2 (top trace) and H-22 (bottom trace) antisera against an ecdysone standard curve. The dashed line shows the gradient elution profile of the analysis. Solvent A, 95% 20 mM Tris perchlorate buffer pH 7.2, 5% acetonitrile. Solvent B, 5% Tris perchlorate buffer, 95% acetonitrile. Waters 15 cm x 4.6 mm int. diam. 5 µm ODS column. Flow rate 1 ml/min.

the Sarcophagidae were noted 24 and 48 h after larviposition at 25°C. These times are similar to those reported by Roberts (1976) for the Australian flesh fly *Tricholioproctia impatiens*, and by Wentworth *et al.* (1981) in *S. bullata*. There appears to be little difference between those lines raised under diapause- and non-diapause-inducing conditions. Following larval exodus from the food however, those larvae raised under short-day conditions wandered for 24 h longer than those raised under long-day conditions. The larval ecdysteroid titres reported here are characterized by a high peak at larviposition, followed by a rapid drop by 12 h. This situation has not been reported previously in *Sarcophaga*. Prior to each

of the two larval moults, a pulse of ecdysteroids is seen. After these ecdyses, the titre remains low in both lines until just before the onset of wandering behaviour when a large pulse of ecdysteroid release occurs. Dominick and Truman (1984) have shown that the onset of wandering in *Manduca sexta* is a circadian-controlled gated event to which the larvae are committed by a release of prothoracicotropic hormone (PTTH), and the accompanying elevation of ecdysteroid levels (Gilbert *et al.*, 1981). Richard *et al.* (1986) have shown that the onset of wandering behaviour in *S. argyrostoma* is also a gated event. Presumably therefore, the increase in haemolymph ecdysteroid titre reported here is in response to an

Table 1. The levels of free specific ecdysteroids detected in diapause (light:dark 12:12) and non-diapause (light:dark 18:6) destined pupae of *Sarcophaga argyrostoma* corrected for cross reactivity with H-22 antiserum. Concentration in ng/ml of haemolymph

Ecdysteroid	Cross reactivity	light:dark 18:6	light:dark 12:12
Ecdysone	1	41	29
20-Hydroxyecdysone	4.5	263	237
20,26-Dihydroxyecdysone	5	34	38
Makisterone A	5	—	8
Highly polar products	1	129	144

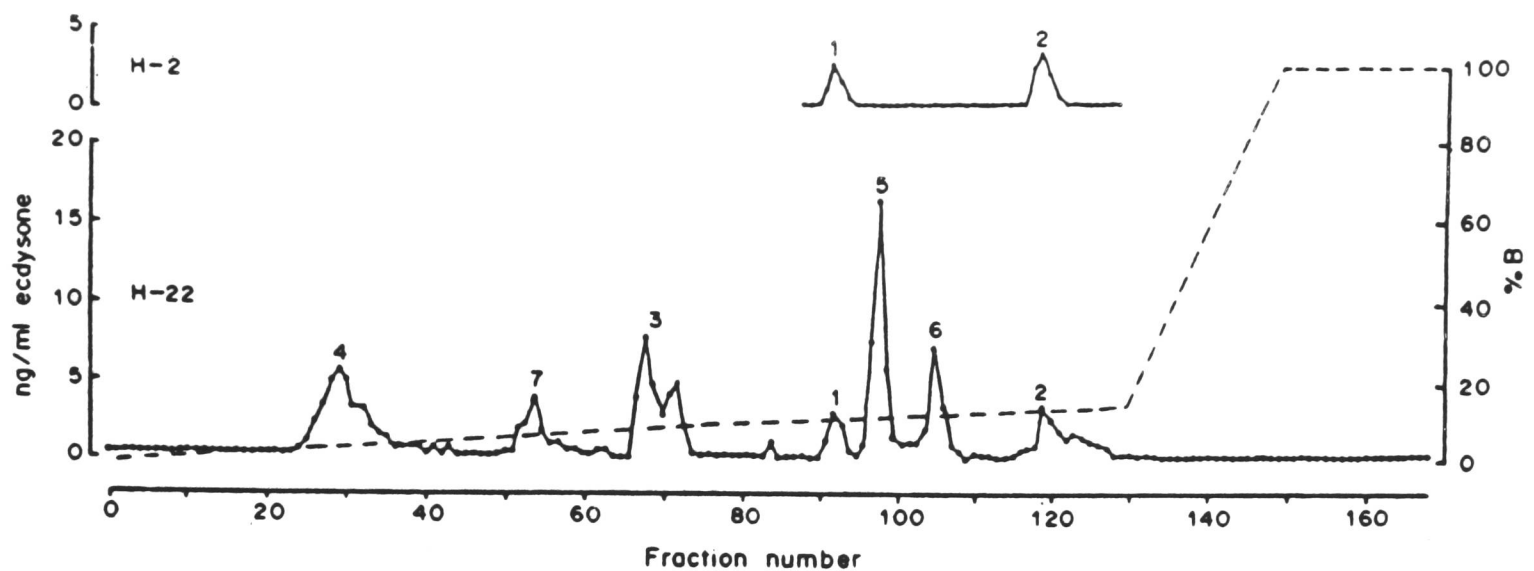


Fig. 5. IS-RPHPLC trace (H-22 antiserum) of ecdysteroids pooled from highly polar products isolated from initial HPLC separations of diapause and non-diapause-destined pupal extracts following deconjugation by a mixture of crude esterases. (1) 20-Hydroxyecdysone; (2) ecdysone; (3) 20,26-dihydroxyecdysone; (4) 20-hydroxyecdysone; (5) 26-hydroxyecdysone; (6) makisterone A; (7) ecdysoneic acid. IS-RPHPLC and RIA conditions as for Fig. 4.

Table 2. The levels of conjugated ecdysteroids and ecdysoneic acids extracted from the pooled highly polar fractions of 4, 8 and 12 h post-pupariation haemolymph from diapause- and non-diapause-destined pupae of *Sarcophaga argyrostoma*

Ecdysteroid	Cross reactivity	Concentration	C/F
Ecdysone	1	12	0.34
20-Hydroxyecdysone	4.5	40	0.16
26-Hydroxyecdysone	1	38	38
20,26-Dihydroxyecdysone	5	186	5.2
Makisterone A	5	108	27
Ecdysoneic acid	1	7	—
20-Hydroxyecdysoneic acid	5	254	—

Results corrected for cross reactivity (CR) with H-22 antiserum. C/F = Ratio of conjugated ecdysteroid to free ecdysteroid. Concentrations in ng/ml of haemolymph.

earlier gated release of PTTH by the brain. There is little documented evidence that ecdysone release prior to larval moulting is gated.

Following wandering, the levels of circulating ecdysteroids are variable. The higher average titre in the non-diapause destined cultures may be associated with the shorter wandering period, in that a higher titre may encourage earlier puparium formation. A development-destined individual will only require a pupation site for approx. 12 days, whereas a diapause-destined insect will require the site for several months during a time of adverse environmental conditions, so a more thorough "selection" of the site may optimize the chances of survival (Denlinger, 1972).

Wentworth *et al.* (1981) showed the presence of a large ecdysteroid peak prior to puparium formation in *S. bullata*. We were only able to show the existence of this peak in the long-day larvae because of problems of developing asynchrony within the short-day cultures by this stage. Unlike the onset of wandering, puparium formation is not a gated event in *S. argyrostoma* (Richard *et al.*, 1986), although Roberts (1984) suggested a weak association with the dark phase in *S. bullata*. In the present investigation therefore, puparia were collected every 4 h to ensure

the synchrony of the timings of the puparial ecdysteroid samples. There were no observed differences in these ecdysteroid titres between the two lines in the first 48 h of intrapuparial development. Both cultures displayed a large peak in the first 24 h of prepupal-pupal development associated with larval-pupal apolysis (Wentworth *et al.*, 1981). Following pupariation, the titres gradually drop below 100 pg/ $\mu$ l after 48 h. The peak noted around 72 h may be associated with the initiation of pupal/adult apolysis (Roberts and Warren, 1975) and may elicit the onset of pharate-adult development. The lower ecdysteroid level in the diapause-destined culture may not be enough to bring about this development. The haemolymph titre in the short day individuals then remained low as they entered diapause, whereas the level of circulating ecdysteroids in the long-day insects increased between 85 and 90 h to a very high level (600 pg/ $\mu$ l), as pharate-adult development commenced. Similar titres were reported by Walker and Denlinger (1980) in *S. crassipalpis* and by Ohtaki and Takahashi (1972) in *S. peregrina* using a *Musca* bioassay technique. Both these groups reported the absence of the adult development peak of ecdysteroids in diapausing flesh flies. The timing of the endocrinological shut down in diapausing pupae of

*S. argyrostoma* is consistent with that reported by these workers and by Roberts and Warren (1975) in *T. impatiens*.

These results support the theory that diapause results from the failure of the brain/prothoracic gland system to effect the production of ecdysone. This is further supported by the observation that diapause in *Sarcophaga* can be terminated by the injection of 20-hydroxyecdysone into diapausing pupae (Gibbs, 1976; Zdarek and Denlinger, 1975). That Fraenkel and Hsiao (1968b) were able to break diapause by the injection of ecdysone, the prohormone to 20-hydroxyecdysone, implies that the enzymatic systems responsible for ecdysone hydroxylation, usually located in the fat body (Gilbert *et al.*, 1980), are either competent to respond, or easily switched on. Bowen *et al.* (1984a) further demonstrated that prothoracic glands from the diapausing pupae of *Manduca sexta* were not as competent to respond to PTTH stimulation *in vitro* as glands from non-diapausing pupae.

The levels of specific ecdysteroids in the haemolymph of diapause- and non-diapause-destined prepupae show few differences between the two lines. Both contain ecdysone, 20-hydroxyecdysone and 20,26-dihydroxyecdysone as the major free ecdysteroids. The highly polar 20-hydroxyecdysoneic acid appears not to have been conjugated as it eluted at the same position after the deconjugation procedures. Other putative free ecdysteroids noted were 26-hydroxyecdysone and makisterone A, although at much lower levels.

The levels of conjugated ecdysteroids determined after enzymatic hydrolysis indicate that conjugation is an important pathway for ecdysone catabolism in *Sarcophaga*. It is generally accepted that ecdysone is hydroxylated by ecdysone 20-monooxygenase to the active form, 20-hydroxyecdysone (Bollenbacher *et al.*, 1977). Bergamasco and Horn (1980) note that 26-hydroxylation, converting ecdysone to 26-hydroxyecdysone, or 20-hydroxyecdysone to 20,26-dihydroxyecdysone, reduces biological activity. Both of these metabolites are found in significant quantities in the conjugate fraction indicating the presence of a 26-monooxygenase, and the subsequent conjugation of these 26-hydroxylated metabolites.

26-hydroxyecdysteroids can be further oxidized to the corresponding carboxylic acids (Koolman, 1982). 26-hydroxyecdysone may be converted directly to ecdysoneic acid which can then be 20-hydroxylated to 20-hydroxyecdysoneic acid. Alternatively, 26-hydroxyecdysone may be first 20-hydroxylated and then oxidized to 20-hydroxyecdysoneic acid. That little ecdysoneic acid was found suggests that the latter route is preferred in *Sarcophaga*. In addition, 20,26-dihydroxyecdysone produced by the 26-hydroxylation of 20-hydroxyecdysone, may also be 26-oxidized to 20-hydroxyecdysoneic acid.

The results show that 26-hydroxyecdysone, 20,26-dihydroxyecdysone and makisterone A are preferentially conjugated over ecdysone and 20-hydroxyecdysone which exist predominantly in the free form. It would appear that the predominant metabolic pathway for ecdysone is hydroxylation to 20-hydroxyecdysone and 26-hydroxyecdysone, the latter then being preferentially conjugated. 20-Hydroxyecdysone appears to be C26-hydroxylated

followed by C26-oxidation to 20-hydroxyecdysoneic acid.

The absence of any observed differences in specific ecdysteroid titres between diapause- and non-diapause-destined lines supports the suggestion that the regulation of diapause induction is probably centred in the brain (Bowen *et al.*, 1985). The most likely system controlling diapause is the regulation of the release of prothoracicotropic hormone, although secondary downstream blocks involving prothoracic gland competency are possible (Bowen *et al.*, 1984).

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